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6/29/99 GB

Int'l. Appln. No.: PCT/EP00/05998
Docket No. B45186

Amendments to the Claims: 15-29

This listing of claims will replace all prior versions, and listings, of claims in the application:

1. – 14. (Cancelled) FRIDE, MARTIN; GARCON, NATHALIE

15. (Previously Presented) A composition for raising an immune response comprising an HIV antigen and an immunostimulatory CpG oligonucleotide.

16. (Previously Presented) A composition as claimed in claim 15 wherein the antigen is selected from the group consisting of HIV antigens, gp120, gp160, Nef, Tat, Nef-Tat fusions and immunologically equivalent derivatives thereof.

17. (Previously Presented) A composition as claimed in claim 15 wherein the antigen is fused to Protein D, or lipoprotein D or a fragment thereof, from Haemophilius influenzae.

18. (Previously Presented) A composition as claimed in claim 15 wherein the antigen comprises a Nef-Tat fusion protein. C. BRUCK

19. (Previously Presented) A composition as claimed in claim 15 further comprising an aluminum salt. (ALUM)

20. (Previously Presented) A composition as claimed in claim 15 further comprising a saponin adjuvant.

21. (Previously Presented) A composition as claimed in claim 15 wherein the oligonucleotide comprises two CpG dinucleotides.

22. (Previously Presented) A composition as claimed in claim 15 wherein the CpG oligonucleotide is between 15-45 nucleotides in length.

23. (Previously Presented) A composition as claimed in claim 15 wherein the CpG oligonucleotide comprises at least one phosphorothioate internucleotide bond.

24. (Currently Amended) A composition as claimed in claim 15 wherein the oligonucleotide is selected from the group consisting of oligonucleotides designated as WD1001

6,544,518
6,558,670
102(b) DATE
5,962,636 → WD1001
6,034,230 → WD1001
6,207,646 * WD1001 KRIEG A.M.
6,339,068 * WD1001 WD1004
KRIEG A.M.
6,406,705 *
7,049,302 *
6,239,116 *
6,429,199

WO 99/16884 Nef-Tat 04/08/99

KRIEG ARTHUR
CpG DINUCLEOTIDES
CpG OLIGO

(SEQ ID NO:1), WD1002 (SEQ ID NO:2), WD1003 (SEQ ID NO:3), WD1004 (SEQ ID NO:4), WD1005 (SEQ ID NO:5), WD1006 (SEQ ID NO:6), and WD1007 (SEQ ID NO:7).

25. (Previously Presented) A method for the prevention or amelioration of HIV infection in a patient, comprising administering an effective amount of a vaccine as claimed in claim 15 to the patient.

26. (Previously Presented) A method for the prevention or amelioration of HIV infection in a patient, comprising administering an effective amount of a vaccine as claimed in claim 19.

27. (Previously Presented) A method for the prevention or amelioration of HIV infection in a patient, comprising administering an effective amount of a vaccine as claimed in claim 20

28. (Previously Presented) A method of producing a vaccine as claimed in claim 15 comprising admixing an HIV antigen and a CpG immunostimulatory oligonucleotide.

29. (Previously Presented) A method for the prevention or amelioration of HIV infection in a patient, comprising administering an effective amount of a CpG oligonucleotide followed after a suitable time by an effective amount of an HIV antigen.

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Amendments to the Specification:

Please replace the paragraph beginning on Page 4, line 22 and continuing on to Page 5 ending at line 12, with the following rewritten paragraph:

-- Preferred oligonucleotides have the following sequences:

	Oligo (internal designation*)	5'-SEQUENCE-3'	CpG	Thio
ODN-1826	WD1001	TCC ATG ACG TTC CTG ACG TT (SEQ ID NO:1)	+	+
ODN-1758	WD1002	TCT CCC AGC GTG CGC CAT (SEQ ID NO:2)	+	+
	WD1003	ACC GAT AAC GTT GCC GGT GAC G (SEQ ID NO:3)	+	-
2, ←	WD1004	G*G*G GTC AAC GTT GAG* G*G*G* G*G (SEQ ID NO:4)	+	Mix
	WD1005	TCC ATG AGC TTC CTG AGC TT (SEQ ID NO:5)	-	+
	WD1006	TCC ATG ACG TTC CTG ACG TT (SEQ ID NO:6)	+	-
	WD1007	ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG	+	+
ODN-2006		TCG TCG TTT TGT CGT TTT GTC GTT (SEQ ID NO:7)	+	+

* alternatively referred to as WD001-WD007

In the above table a + in the Thio column indicates the presence of a thioate modification. 'Mix' indicates a mixture of thioate modification and sequence without thioate modification (the asterisks indicate the linkages with a thioate modification). A – in the Thio column indicates absence of a thioate modification. A + in the CpG column indicates a the presence of a CpG motif and a – in the CpG column indicates absence of a CpG motif. For example WD1005 contains a GpC rather than a CpG motif, thus it is marked with a - in the CpG column of the table. WD1007 contains a palindromic motif (GACGTC) as well as other non-palindromic CpG sequences. This is also within the scope of a CpG oligonucleotide as the term is used in the present application.--

US006429199B1

(12) **United States Patent**
Krieg et al.

(10) **Patent No.: US 6,429,199 B1**
(45) **Date of Patent: Aug. 6, 2002**

(54) **IMMUNOSTIMULATORY NUCLEIC ACID MOLECULES FOR ACTIVATING DENDRITIC CELLS**

(75) Inventors: **Arthur M. Krieg**, Iowa City, IA (US); **Gunther Hartmann**, München (DE)

(73) Assignee: **University of Iowa Research Foundation**, Iowa City, IA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/191,170**

(22) Filed: **Nov. 13, 1998**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/960,774, filed on Oct. 30, 1997, now Pat. No. 6,239,116, which is a continuation-in-part of application No. 08/738,652, filed on Oct. 30, 1996, now Pat. No. 6,207,646, which is a continuation-in-part of application No. 08/386,063, filed on Feb. 7, 1995, now Pat. No. 6,194,388, which is a continuation-in-part of application No. 08/276,358, filed on Jul. 15, 1994, now abandoned.

(51) **Int. Cl.**[7] **A61P 37/04**

(52) **U.S. Cl.** **514/44**

(58) **Field of Search** 514/44

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WO	WO 96/35782		11/1996
WO	WO 97/12633		4/1997
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(List continued on next page.)

Primary Examiner—James Martinell
(74) *Attorney, Agent, or Firm*—Wolf, Greenfield & Sacks, P.C.

(57) **ABSTRACT**

The present invention relates generally to methods and products for activating dendritic cells. In particular, the invention relates to oligonucleotides which have a specific sequence including at least one unmethylated CpG dinucleotide which are useful for activating dendritic cells. The methods are useful for in vitro, ex-vivo, and in vivo methods such as cancer immunotherapeutics, treatment of infectious disease and treatment of allergic disease.

21 Claims, 13 Drawing Sheets

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FIG. 1

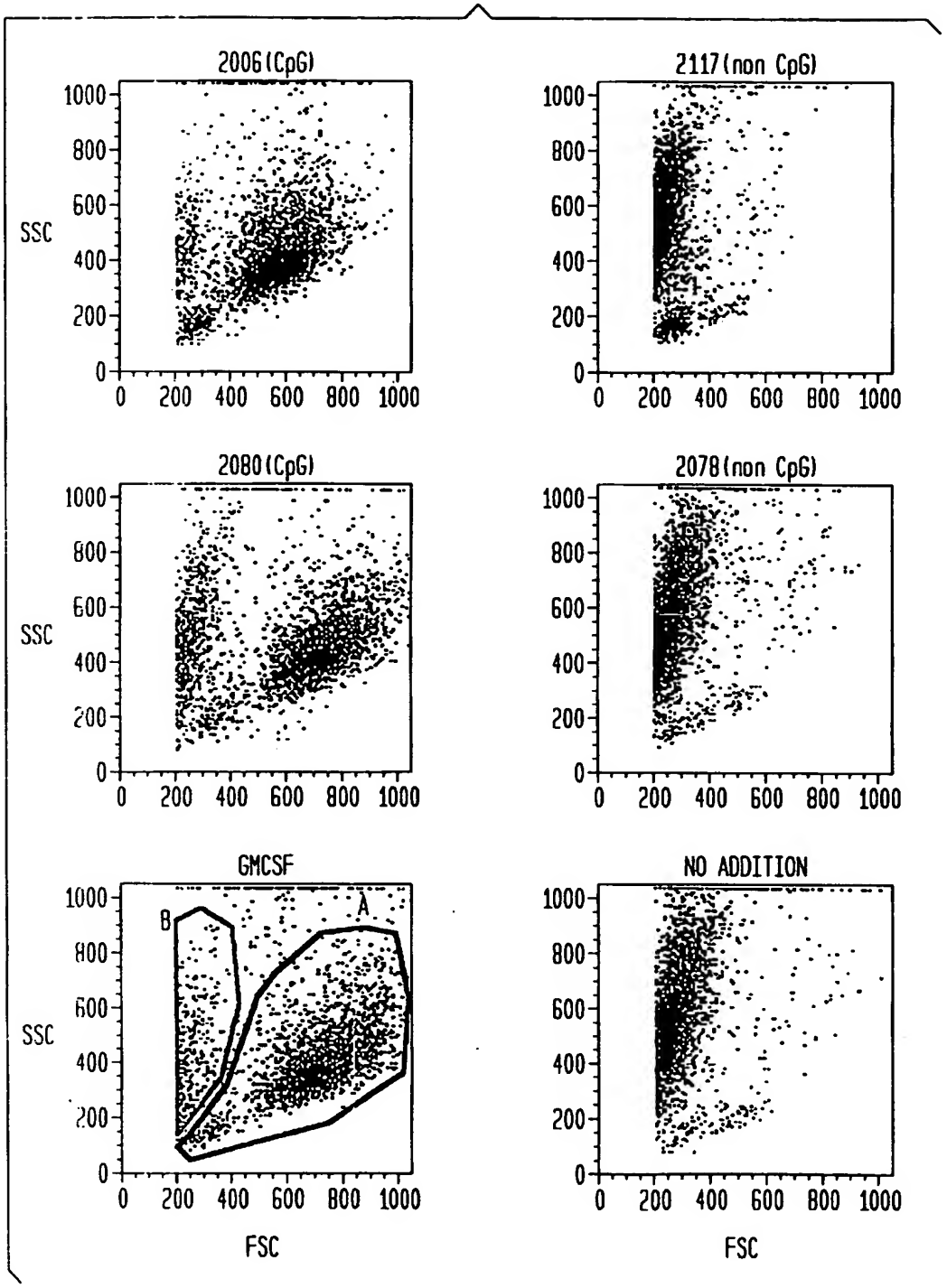

2006(CpG)
2117(non CpG)
2080(CpG)
2078(non CpG)
GMCSF
NO ADDITION
SSC
FSC
A
B
0
200
400
600
800
1000

FIG. 2

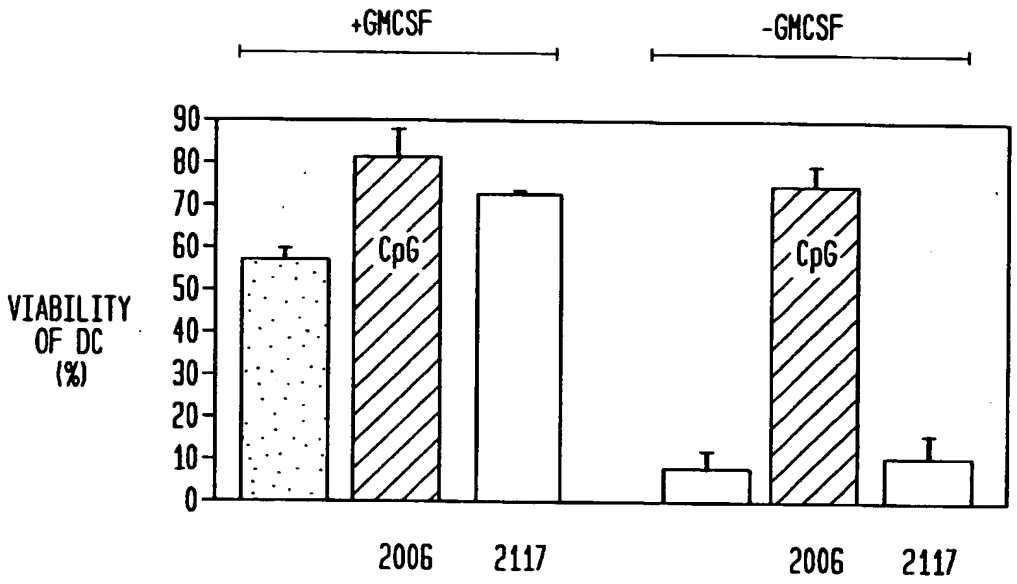

+GMCSF
-GMCSF
VIABILITY OF DC (%)
90
80
70
60
50
40
30
20
10
0
CpG
CpG
2006
2117
2006
2117

FIG. 3

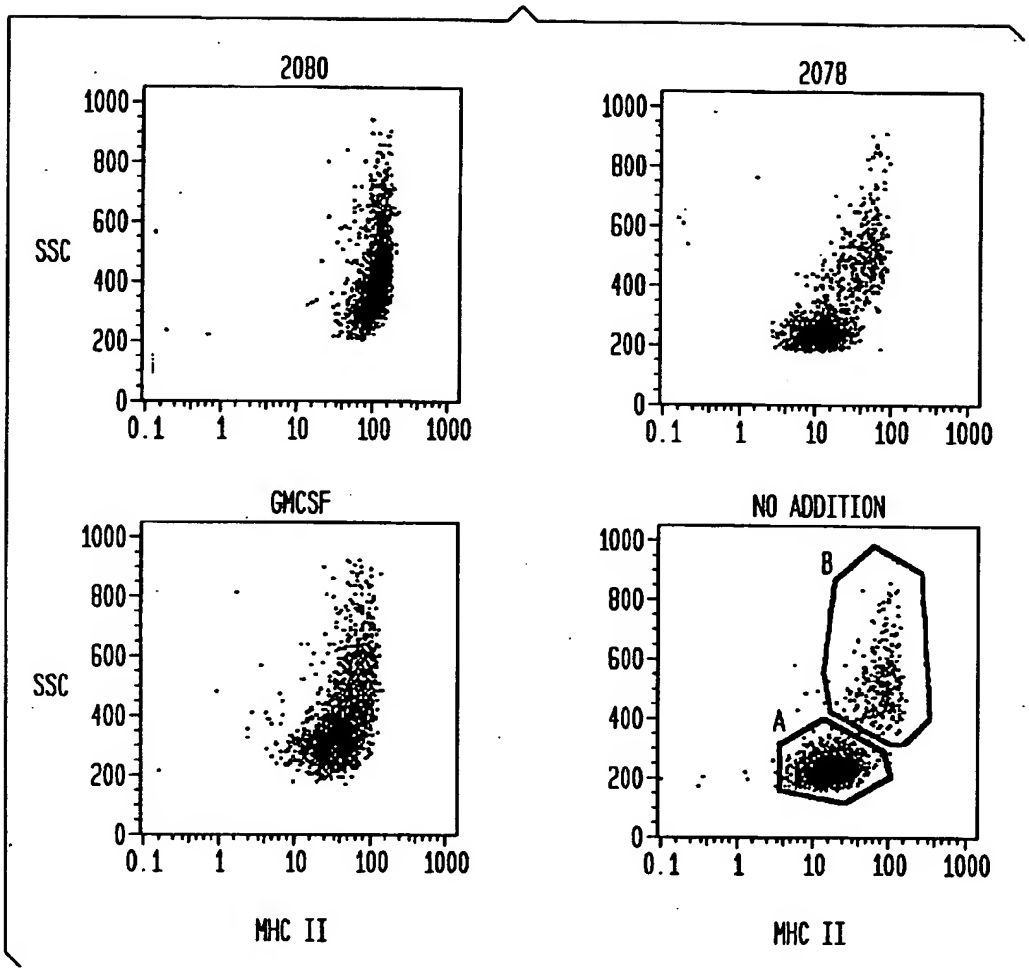

2080
2078
GMCSF
NO ADDITION
SSC
SSC
MHC II
MHC II
A
B
0
200
400
600
800
1000
0.1
1
10
100
1000

FIG. 4

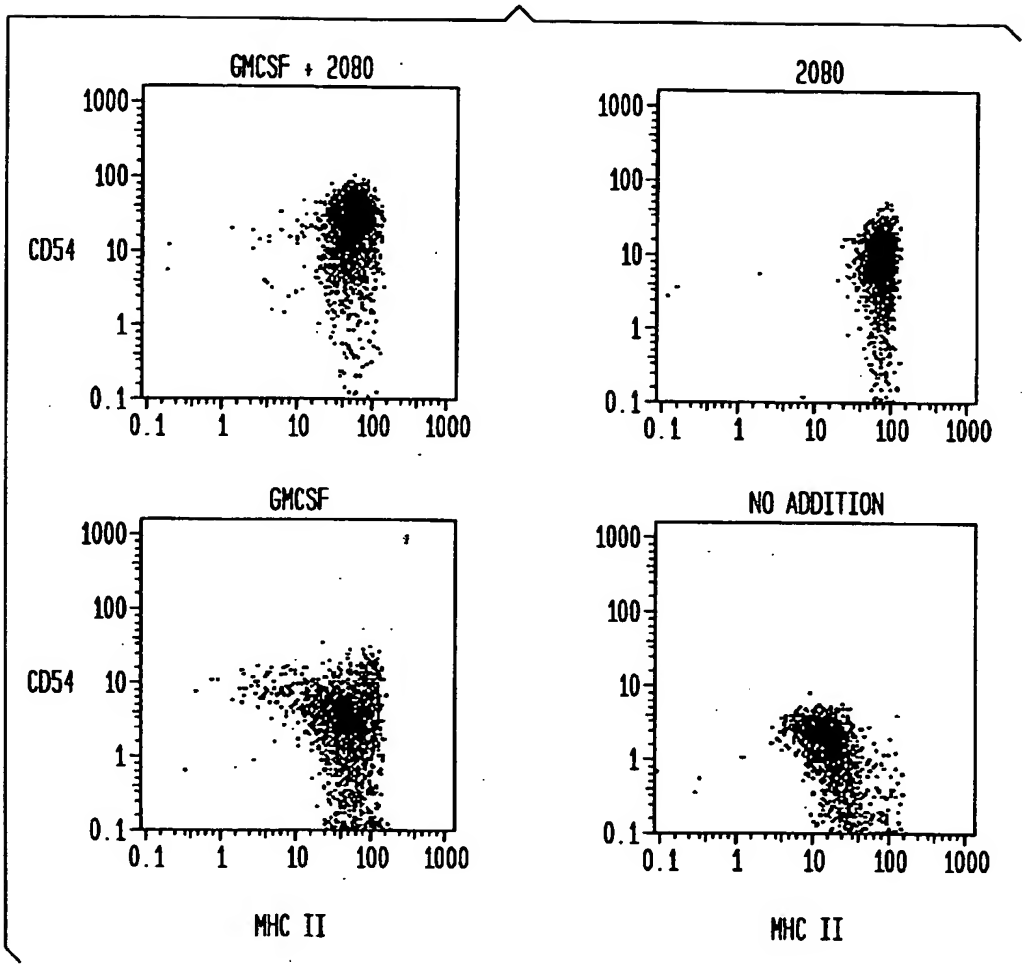

GMCSF + 2080
2080
GMCSF
NO ADDITION
CD54
MHC II
1000
100
10
1
0.1
0.1
1
10
100
1000

FIG. 5A

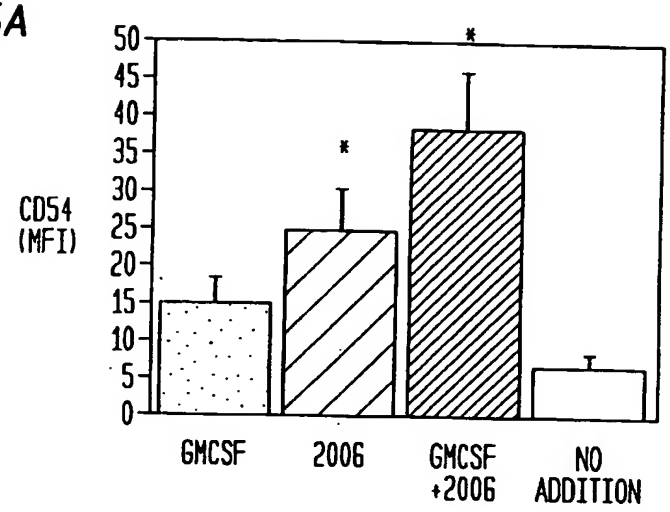


FIG. 5B

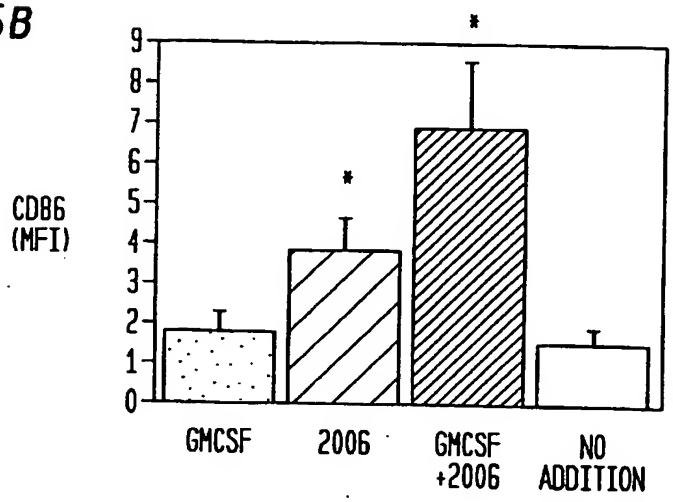


FIG. 5C

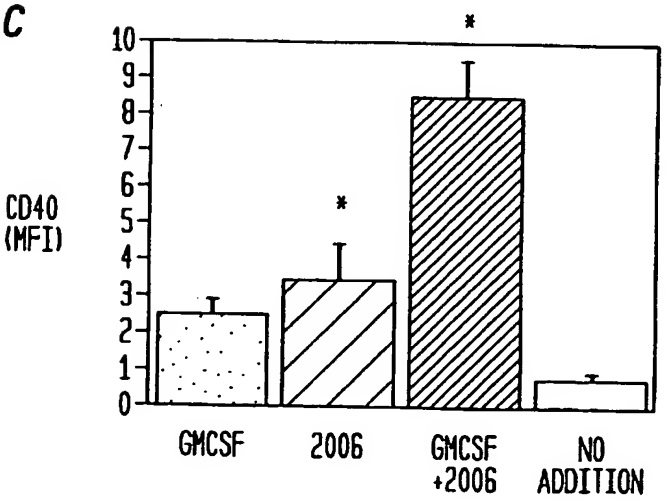


FIG. 6A

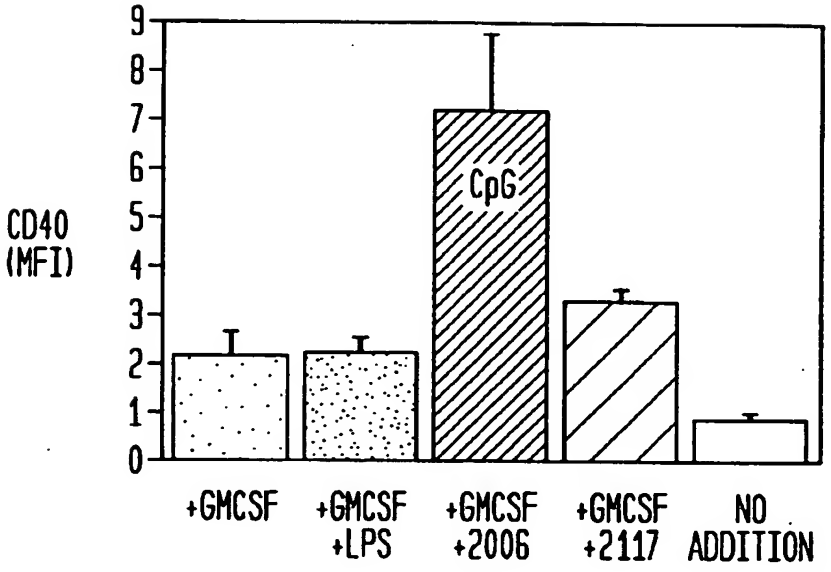

CD40
(MFI)
9
8
7
6
5
4
3
2
1
0
CpG
+GMCSF
+GMCSF
+LPS
+GMCSF
+2006
+GMCSF
+2117
NO
ADDITION

FIG. 6B

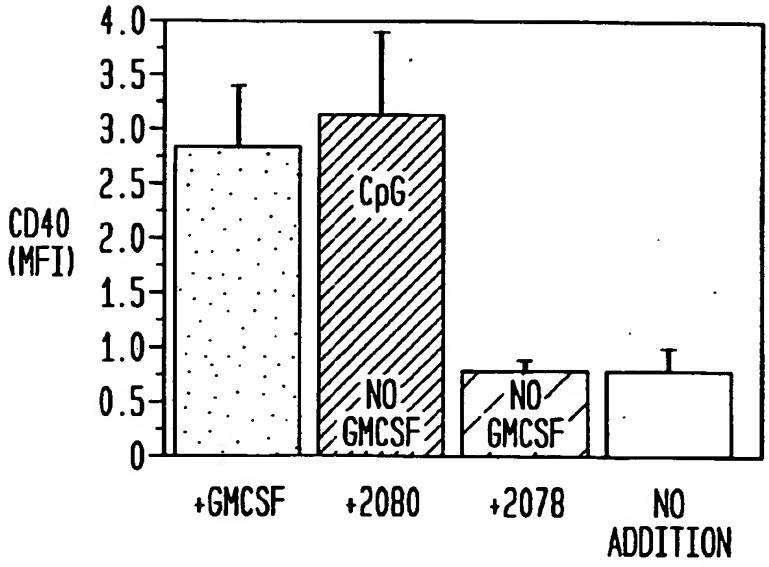

CD40
(MFI)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
CpG
NO
GMCSF
NO
GMCSF
+GMCSF
+2080
+2078
NO
ADDITION

FIG. 7A

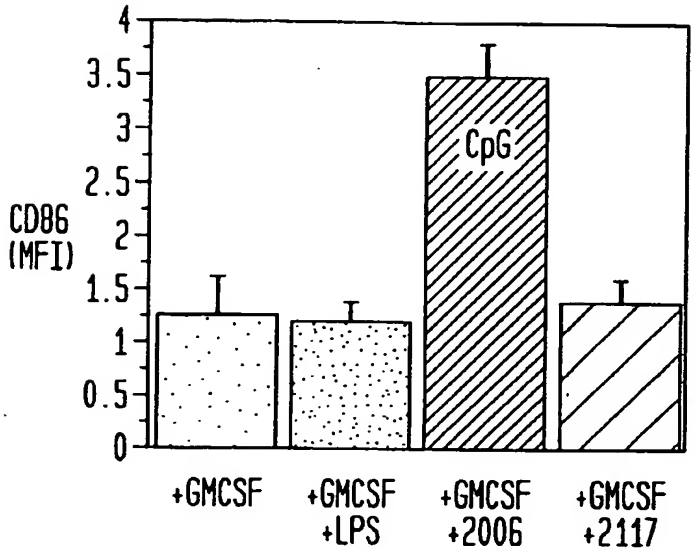

CD86
(MFI)
4
3.5
3
2.5
2
1.5
1
0.5
0
CpG
+GMCSF
+GMCSF
+LPS
+GMCSF
+2006
+GMCSF
+2117

FIG. 7B

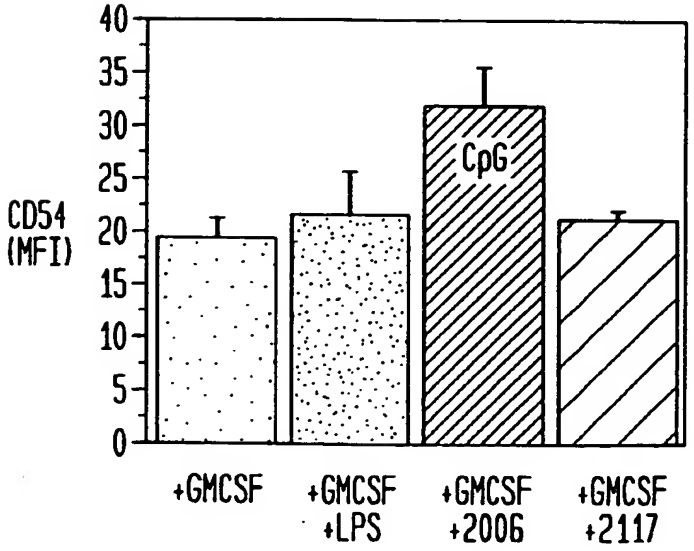

CD54
(MFI)
40
35
30
25
20
15
10
5
0
CpG
+GMCSF
+GMCSF
+LPS
+GMCSF
+2006
+GMCSF
+2117

FIG. 8

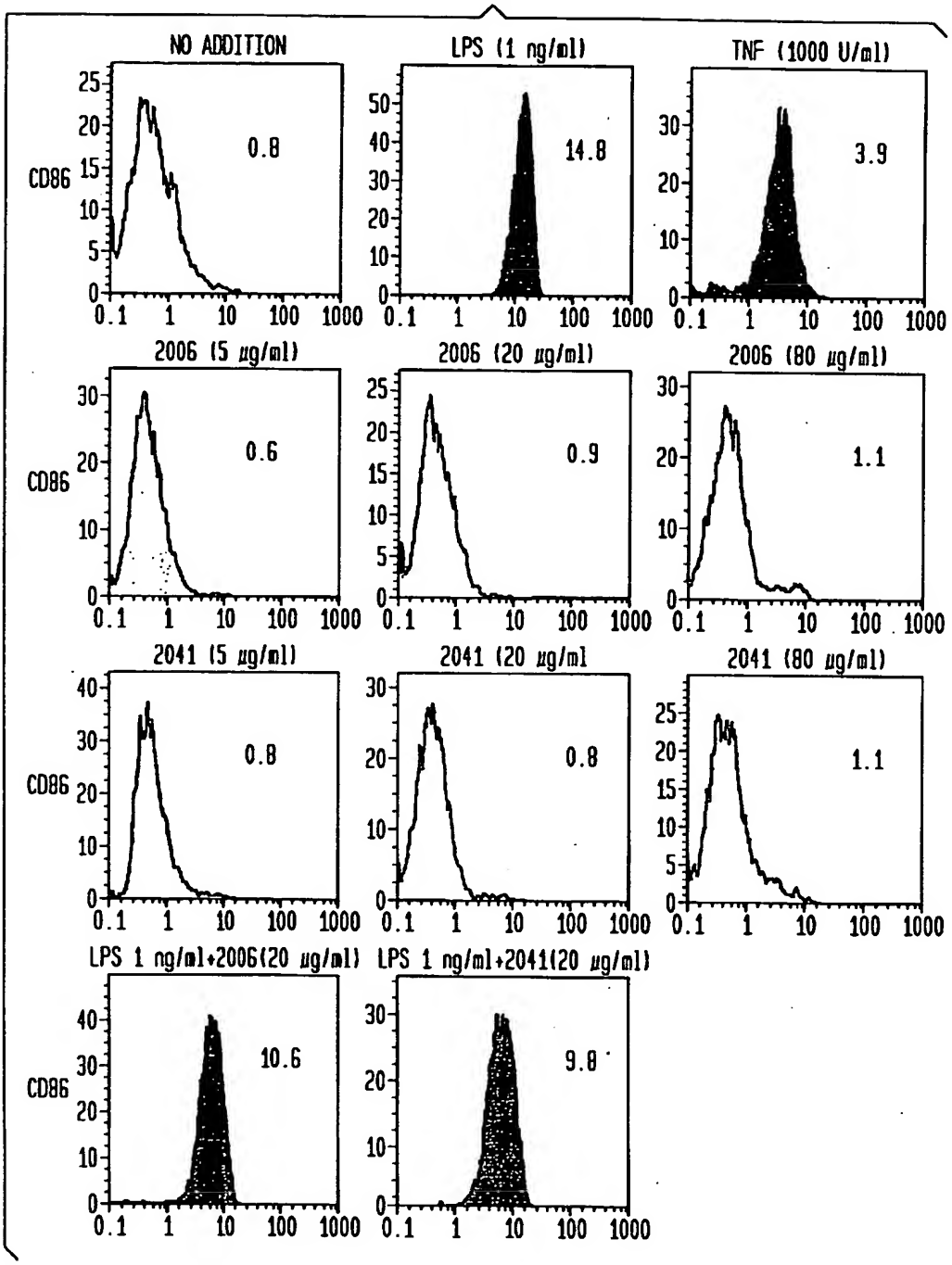

NO ADDITION
LPS (1 ng/ml)
TNF (1000 U/ml)
2006 (5 μg/ml)
2006 (20 μg/ml)
2006 (80 μg/ml)
2041 (5 μg/ml)
2041 (20 μg/ml
2041 (80 μg/ml)
LPS 1 ng/ml+2006(20 μg/ml)
LPS 1 ng/ml+2041(20 μg/ml)
CD86
0.8
14.8
3.9
0.6
0.9
1.1
0.8
0.8
1.1
10.6
9.8
0.1
1
10
100
1000

FIG. 9

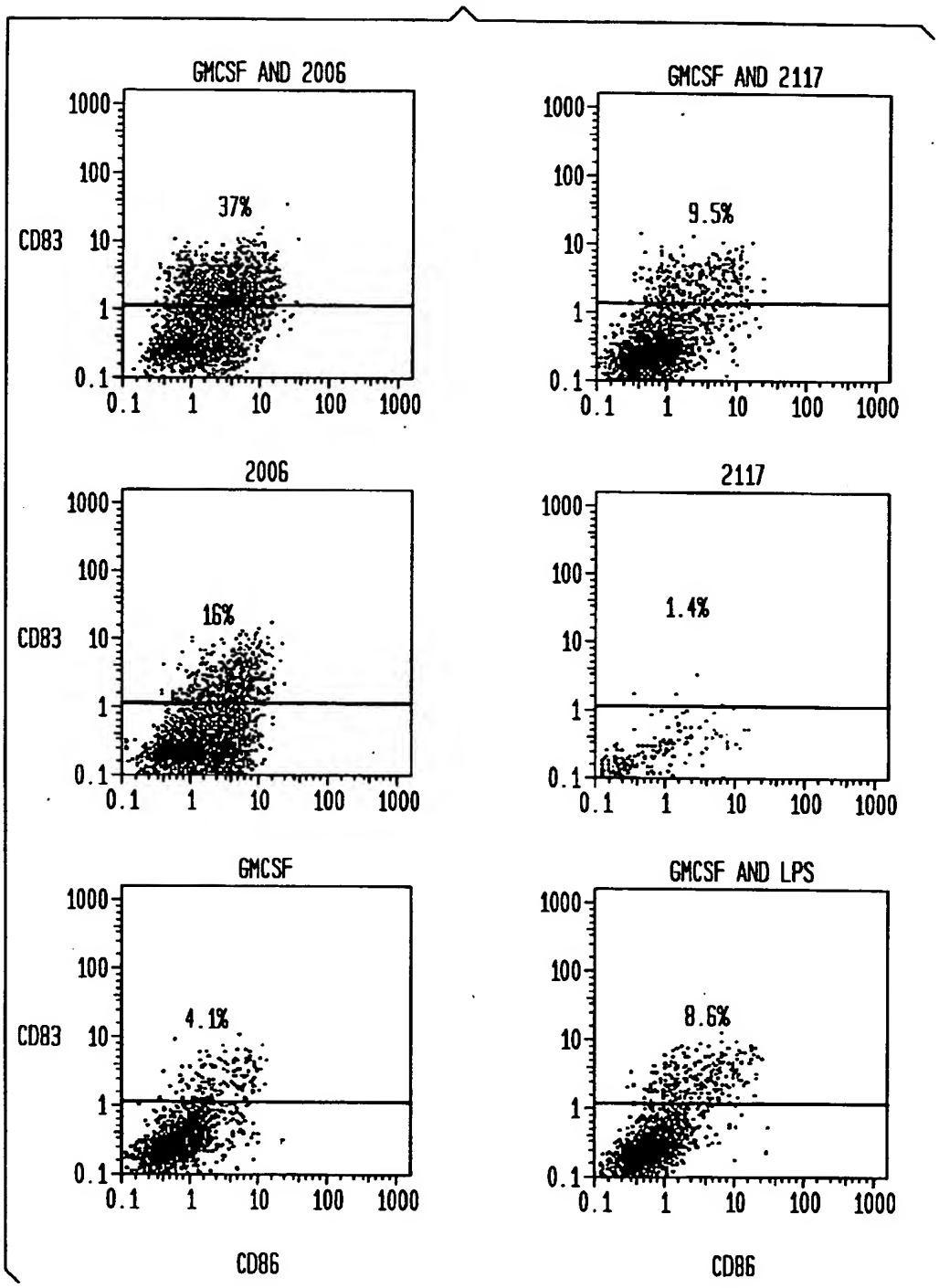

GMCSF AND 2006
37%
GMCSF AND 2117
9.5%
2006
16%
2117
1.4%
GMCSF
4.1%
GMCSF AND LPS
8.6%
CD83
CD86
0.1
1
10
100
1000

FIG. 10A

GMCSF + CpG

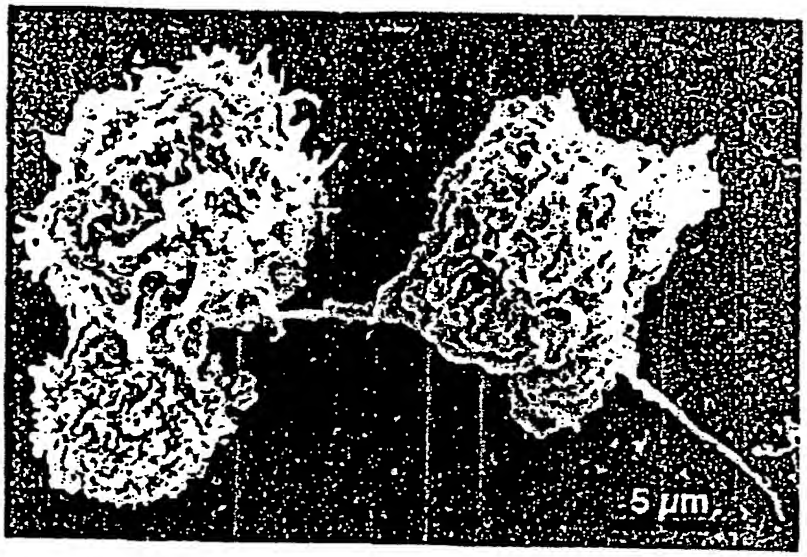


FIG. 10B

CpG

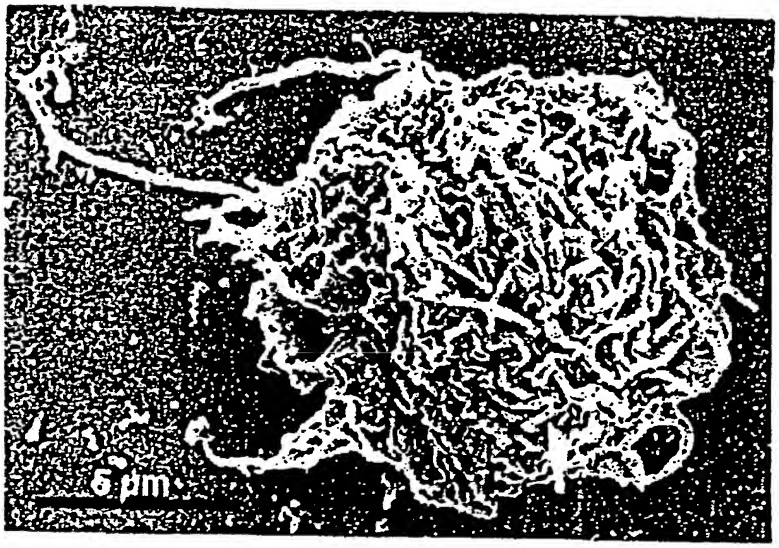


FIG. 10C

GMCSF

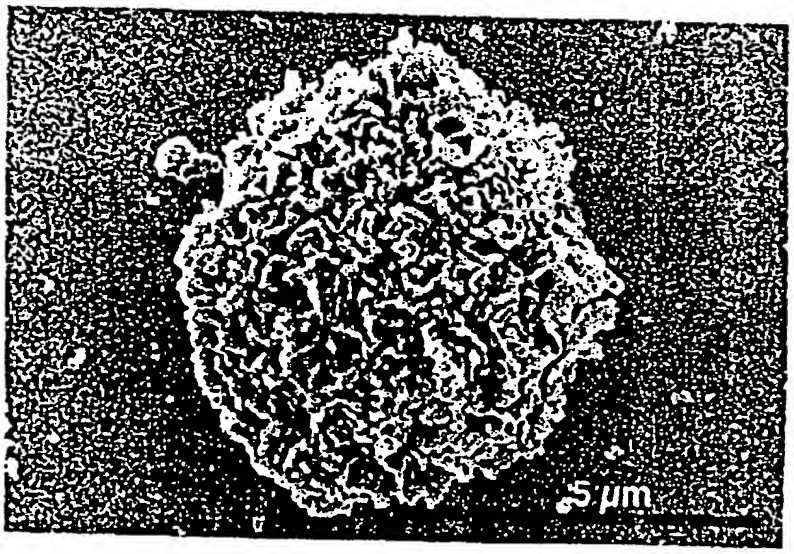


FIG. 10D

GMCSF + non-CpG

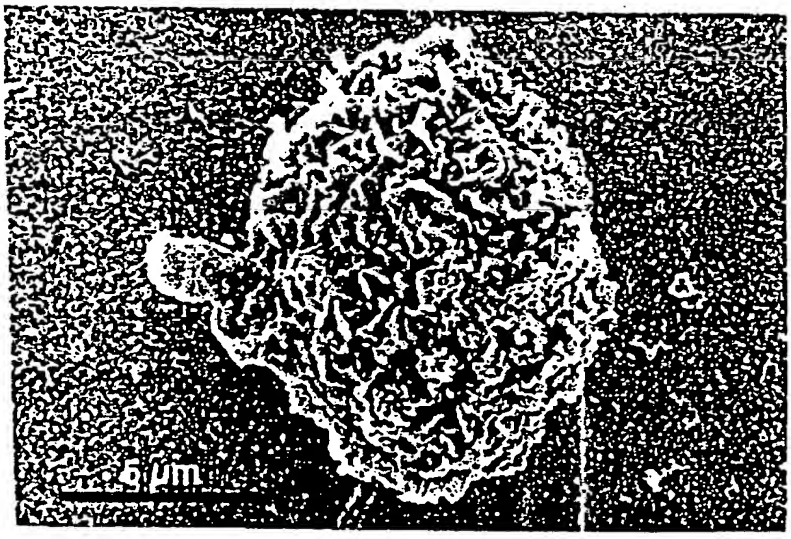


FIG. 11A

+CpG

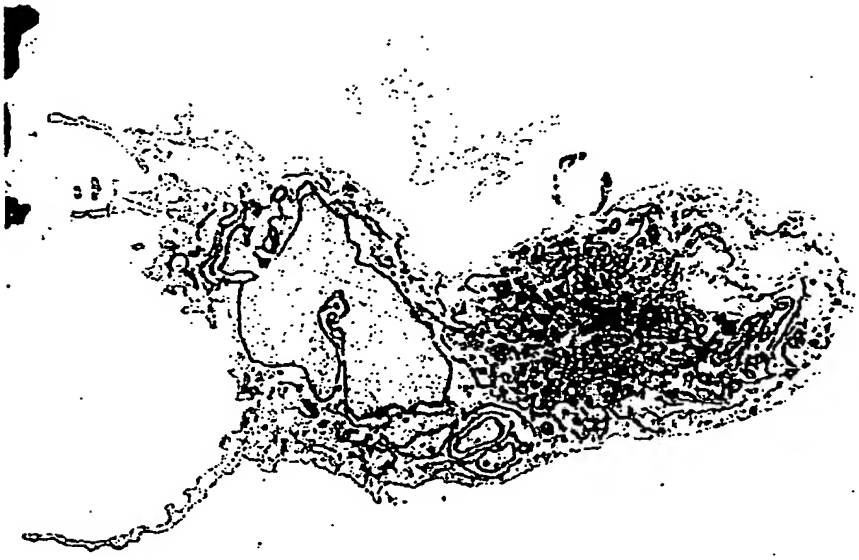


FIG. 11B

-CpG

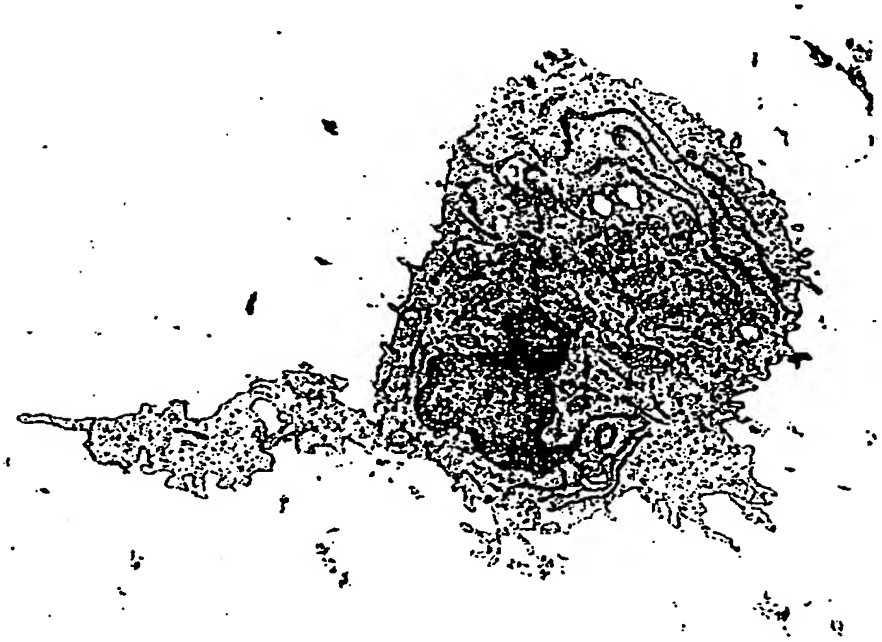


FIG. 12A

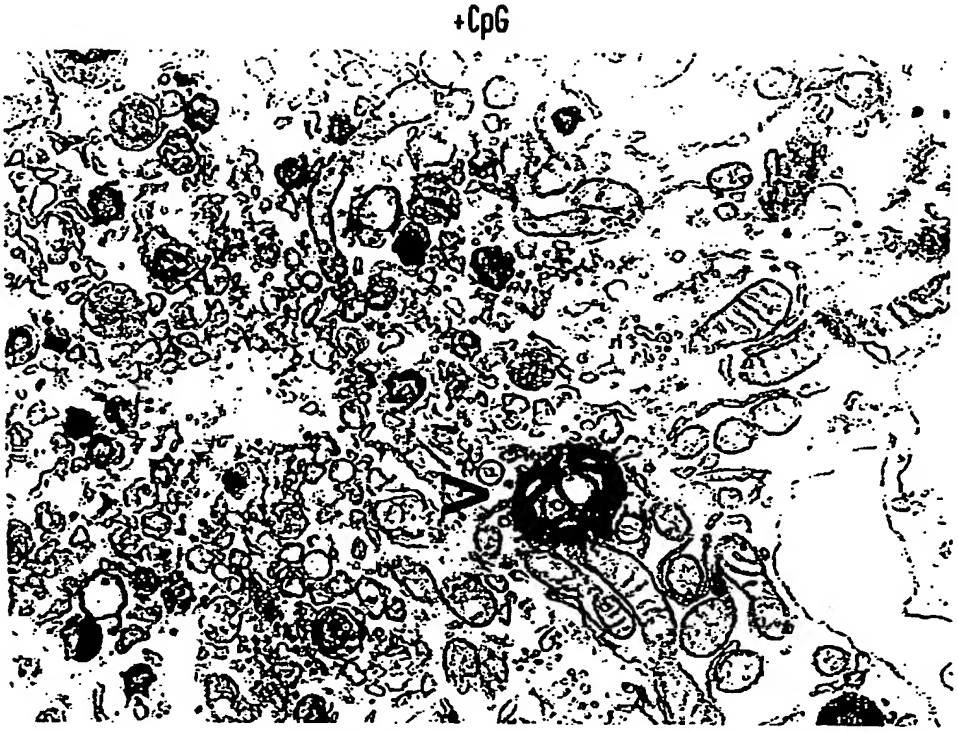
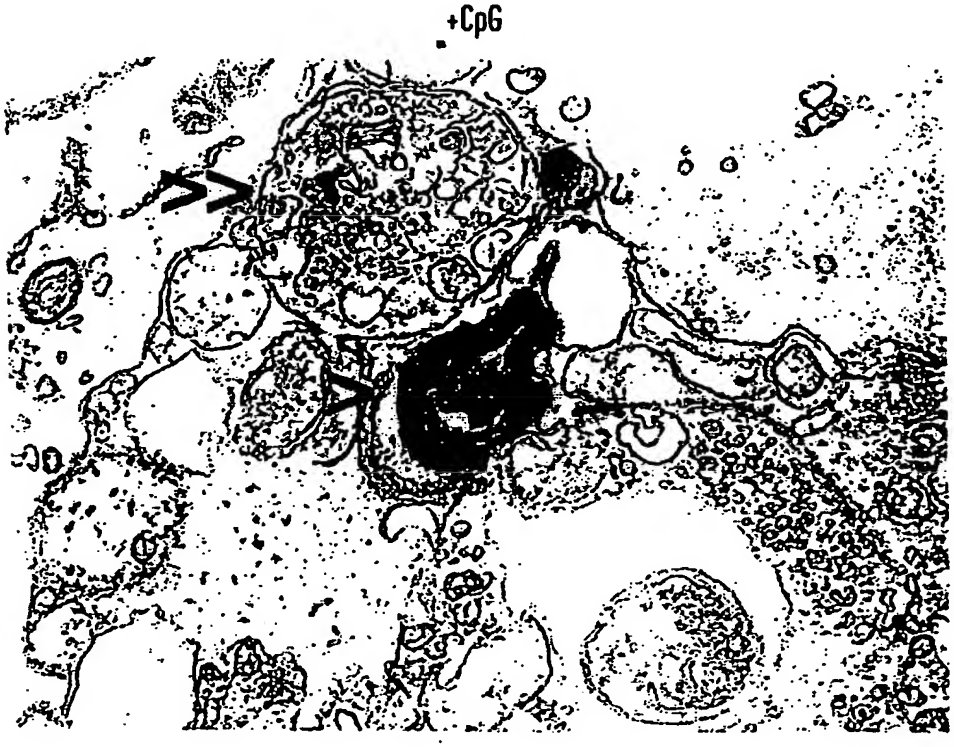


FIG. 12B



IMMUNOSTIMULATORY NUCLEIC ACID MOLECULES FOR ACTIVATING DENDRITIC CELLS

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Ser. No. 08/960,774, filed Oct. 30, 1997 now U.S. Pat. No. 6,239,116, which is a continuation-in-part of U.S. Ser. No. 08/738,652, filed Oct. 30, 1996 and now U.S. Pat. No. 6,207,646, and which application is a continuation-in-part of U.S. Ser. No. 08/386,063, filed Feb. 7, 1995 and now U.S. Pat. No. 6,194,388, and which application is a continuation-in-part of U.S. Ser. No. 08/276,358, filed Jul. 15, 1994 and which is abandoned.

FIELD OF THE INVENTION

The present invention relates generally to methods and products for activating dendritic cells. In particular, the invention relates to oligonucleotides which have a specific sequence including at least one unmethylated CpG dinucleotide which are useful for activating dendritic cells.

BACKGROUND OF THE INVENTION

In the 1970s, several investigators reported the binding of high molecular weight DNA to cell membranes (Lerner, R. A., et al., 1971, *Proc. Natl. Acad. Sci. USA*, 68:1212; Aggarwal, S. K., et al., 1975, *Proc. Natl. Acad. Sci. USA*, 72:928). In 1985, Bennett et al. presented the first evidence that DNA binding to lymphocytes is similar to a ligand receptor interaction; binding is saturable, competitive, and leads to DNA endocytosis and degradation into oligonucleotides (Bennett, R. M., et al., *J. Clin. Invest.*, 76:2182). Like DNA, oligodeoxyribonucleotides (ODNs) are able to enter cells in a saturable, sequence independent, and temperature and energy dependent fashion (reviewed in Jaroszewski, J. W., et al. and J. S. Cohen, 1991, *Advanced Drug Delivery Reviews*, 6:235; Akhtar, et al., 1992, in: "Gene Regulation: Biology of Antisense RNA and DNA," R. P. Erickson, Eds, Raven Press, Ltd., New York, p. 133; and Zhao, et al., 1994, Blood, 84:3660). No receptor for DNA or ODN uptake has yet been cloned, and it is not yet clear whether ODN binding and cell uptake occurs through the same or a different mechanism from that of high molecular weight DNA.

Lymphocyte ODN uptake has been shown to be regulated by cell activation. Spleen cells stimulated with the B cell mitogen LPS had dramatically enhanced ODN uptake in the B cell population, while spleen cells treated with the T cell mitogen ConA showed enhanced ODN uptake by T but not B cells (Krieg, A. M., et al., 1991, *Antisense Research and Development*, 1:161).

Several polynucleotides have been extensively evaluated as biological response modifiers. Perhaps the best example is poly(IC) which is a potent inducer of interferon (IFN) production as well as a macrophage activator and inducer of NK activity (Talmadge, J. E., et al., 1985, *Cancer Res.*, 45:1058; Wiltrout, et al., 1985, *J. Biol. Resp. Mod.*, 4:512; Krown, S. E., 1986, *Sem. Oncol.*, 13:207; and Ewel, C. H., et al., 1992, Canc. Res., 52:3005). It appears that this murine NK activation may be due solely to induction of IFN-β secretion (Ishikawa, R., and C. A. Biron, 1993, *J. Immunol.*, 150:3713). This activation was specific for the ribose sugar since deoxyribose was ineffective. Its potent in vitro antitumor activity led to several clinical trials using poly(IC) complexed with poly-L-lysine and carboxymethylcellulose (to reduce degradation by RNAse) (Talmadge, et al., cited supra; Wiltrout, et al., cited supra; Krown, et al., cited supra, and Ewel, et al., cited supra). Unfortunately, toxic side effects has thus far prevented poly(IC) from becoming a useful therapeutic agent.

Guanine ribonucleotides substituted at the C8 position with either a bromine or a thiol group are B cell mitogens and may replace "B cell differentiation factors" (Feldbush, T. L., and Z. K. Ballas, 1985, *J. Immunol.*, 134:3204; and Goodman, M. J., 1986, *J. Immunol.*, 136:3335). 8-mercaptoguanosine and 8-bromoguanosine also can substitute for the cytokine requirement for the generation of MHC restricted CTL (Feldbush, T. L., cited supra), augment murine NK activity (Koo, G.C., et al., 1988, *J. Immunol.*, 140:3249) and synergize with IL-2 in inducing murine LAK generation (Thompson, R. A., and Z. K. Ballas, 1990, *J. Immunol.*, 145:3524). The NK and LAK augmenting activities of these C8-substituted guanosines appear to be due to their induction of IFN (Thompson, cited supra). Recently a 5' triphosphorylated thymidine produced by a mycobacterium was found to be mitogenic for a subset of human γδ T cells (Constant, P., et al., 1994, *Science*, 264:267). This report indicated the possibility that the immune system may have evolved ways to preferentially respond to microbial nucleic acids.

Several observations suggest that certain DNA structures may also have the potential to activate lymphocytes. For example, Bell, et al. reported that nucleosomal protein-DNA complexes (but not naked DNA) in spleen cell supernatants caused B cell proliferation and immunoglobulin secretion (Bell, D. A., et al., 1990, *J. Clin. Invest.*, 85:1487). In other cases, naked DNA has been reported to have immune effects. For example, Messina, et al. have recently reported that 260–800 bp fragments of poly(bG).(dC) and poly(dG, dC) were mitogenic for B cells (Messina, J. P., et al., 1993, *Cell. Immunol.*, 147:148). Tokunaga, et al. have reported that poly(dg, dc) induces the γ-IFN and NK activity (Tokunaga, et al., 1988, *Jpn. J Cancer Res.*, 79:682). Aside from such artificial homopolymer sequences, Pisetsky, et al. reported that pure mammalian DNA has no detectable immune effects, but that DNA from certain bacteria induces B cell activation and immunoglobulin secretion (Messina, et al., 1991, *J. Immunol.*, 147:1759). Assuming that these data did not result from some unusual contaminant, these studies suggested that a particular structure or other characteristic of bacterial DNA renders it capable of triggering B cell activation. Investigations of microbacterial DNA sequences have demonstrated that ODN, which contains certain palindrome sequences can activate NK cells (Yamamoto, et al., 1992, *J. Immunol.*, 148:4072; and Kuramoto, et al., 1992, *Jpn. J. Cancer Res.*, 83:1128).

Several phosphorothioate modified ODN have been reported to induce in vitro or in vivo B cell stimulation (Tanaka, et al., 1992, *J. Exp. Med.*, 175:597; Branda, R. S., et al., 1993, *Biochem. Pharmacol.*, 45:2037; McIntyre, K., et al., 1993, *Antisense Res. Develop.*, 3:309; and Pisetesky, et al., 1994, *Life Sciences*, 54:101). These reports do not suggest a common structure motif or sequence element in these ODN that might explain their effects.

Dendritic cells are considered to be the most potent professional antigen-presenting cells (APC) (Guery, J. C., et al., 1995, *J. Immunol.*, 154:536). Dendritic cells capture antigen and present them as peptide fragments to T cells, stimulating T cell dependent immunity. These powerful APCs have been found in skin, blood, dense tissue, and mucosa, and spleen. Several studies have demonstrated that after human dendritic cells which are isolated from peripheral blood are presented peptide antigen they can be used to

stimulate and expand antigen specific CD4+ and CD8+ T cells, in vitro and ex vivo (Engleman, E. G., 1997, *Cytotechnology*, 25:1). Several clinical trials are currently underway, based on these findings, using ex vivo manipulation of dendritic cells to generate specific anti-tumor dendritic cells for reimplantation. There has been a growing interest in using dendritic cells ex vivo as tumor or infectious disease vaccine adjuvants (Nestle OF, et al., "Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells", *Nat Med*, 1998; 4: 328–332; Rosenberg S A, et al., "Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma", *Nat Med*, 1998; 4:321–327; Hsu F J, et al., "Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells", *Nat Med*, 1996; 2: 52–58; Tjoa BA, et al., "Evaluation of phase I/II clinical trials in prostate cancer with dendritic cells and PSMA peptides", *Prostate*, 1998; 36: 39–44. Numerous animal models demonstrate conclusively that ex vivo generated DC pulsed with protein antigen can be successfully applied for the immunotherapy of cancer and infectious diseases. (Fields R C, et al., "Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo", *Proc Natl Acad Sci, USA*, 1998; 95: 9482–9487; Okada H, et al., "Bone marrow-derived dendritic cells pulsed with a tumor-specific peptide elicit effective anti-tumor immunity against intracranial neoplasms", *Int J Cancer*, 1998; 78: 196–201; Su H, et al., "Vaccination against chlamydial genital tract infection after immunization with dendritic cells pulsed ex vivo with nonviable Chlamydiae", *J Exp Med*, 1998; 188: 809–818; DeMatos P., et al., "Pulsing of dendritic cells with cell lysates from either B16 melanoma or MCA-106 fibrosarcoma yields equally effective vaccines against B 16 tumors in mice", *J Surg Oncol*, 1998; 68: 79–91; Yang S, et al., "Immunotherapeutic potential of tumor antigen-pulsed and unpulsed dendritic cells generated from murine bone marrow", *Cell Immunol*, 1997; 179: 84–95; Nair S K, et al., "Regression of tumors in mice vaccinated with professional antigen- presenting cells pulsed with tumor extracts", *Int J Cancer*, 1997; 70: 706–715.

SUMMARY OF THE INVENTION

As described in co-pending parent patent application U.S. Ser. No. 08/960,774 the vertebrate immune system has the ability to recognize the presence of bacterial DNA based on the recognition of so-called CpG-motifs, unmethylated cytidine-guanosine dinucleotides within specific patterns of flanking bases. According to these disclosures CpG functions as an adjuvant and is as potent at inducing B-cell and T-cell responses as the complete Freund's adjuvant, but is preferable since CpG induces a higher Th1 response and is less toxic. Alum, the adjuvant which is used routinely in human vaccination, induces the less favorable Th2 response. Compared to alum, CpG is a more effective adjuvant. The combination of CpG and alum was found to produce a synergistic adjuvant effect.

CpG oligonucleotides also show adjuvant effects towards various immune cells. For instance, CpG enhances the efficacy of monoclonal antibody therapy, thus functioning as an effective immune adjuvant for antigen immunization in a B cell lymphoma model. Cytotoxic T cell responses to protein antigen also are induced by CpG. Furthermore, the presence of immunostimulatory DNA sequences in plasmids was found to be necessary for effective intradermal gene immunization.

It was discovered according to an aspect of the invention that the adjuvant activity of CpG is based on the direct activation of dendritic cells by CpG. Potent immunostimulatory CpG oligonucleotides and control oligonucleotides were found to cause dramatic changes in dendritic cells isolated from peripheral blood by immunomagnetic cell sorting. CpG oligonucleotides provided excellent Dendritic cell survival, differentiation, activation and maturation, and were superior to the combination of GM-CSF and LPS. In fact, the combination of CpG and GM-CSF produced unexpected synergistic effects on the activation of dendritic cells. The invention thus encompasses both CpG oligonucleotides and the combination of CpG oligonucleotides and cytokines such as GM-CSF as well as in vitro, ex vivo, and in vivo methods of activating dendritic cells for various assays and immunotherapeutic strategies.

In one aspect the invention is a method for activating a dendritic cell. The method includes the steps of contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to activate a dendritic cell. In one embodiment the dendritic cell is an isolated dendritic cell.

The isolated nucleic acid is one which contains at least one unmethylated CpG dinucleotide and which is from about 8–80 bases in length. In one embodiment the unmethylated CpG dinucleotide has a formula:

$$5'N_1X_1CGX_2N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0–25 nucleotides. In another embodiment the unmethylated CpG dinucleotide has a formula:

$$5'N_1X_1X_2CGX_3X_4N3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of TpT, CpT, TpC, and ApT; X_3X_4 is selected from the group consisting of GpT,GpA, ApA and ApT; N is any nucleotide and N_1+N_2 is from about 0–25 nucleotides. In a preferred embodiment N_1 and N_2 of the nucleic acid do not contain a CCGG quadmer or more than one CCG or CGG trimer. In an illustrative embodiment the isolated nucleic acid is selected from the group consisting of SEQ ID NOS. 20, 24, and 38–46. In another embodiment the isolated nucleic acid is SEQ ID NO.: 84 or 85.

In yet another embodiment the nucleotide of the isolated nucleic acid has a phosphate backbone modification, such as, for example, a phosphorothioate or phosphorodithioate modification. In one embodiment the phosphate backbone modification occurs at the 5' end of the nucleic acid. Preferably the phosphate backbone modification occurs at the first two internucleotide linkages of the 5' end of the nucleic acid. According to another embodiment the phosphate backbone modification occurs at the 3' end of the nucleic acid. Preferably, the phosphate backbone modification occurs at the last five internucleotide linkages of the 3' end of the nucleic acid.

The method for activating the dendritic cell may be performed in vitro, ex vivo, or in vivo. The method in some aspects is a method for cancer immunotherapy, treating an infectious disease, or treating an allergy. When these methods are performed ex vivo they are performed by administering an activated dendritic cell that expresses a specific cancer antigen, microbial antigen or allergen to a subject in need thereof, wherein the activated dendritic cell is prepared by the methods described above. In a preferred embodiment the isolated nucleic acid is administered to a human subject.

In other embodiments the method includes the step of contacting the dendritic cell with a cytokine selected from the group consisting of GM-CSF, IL-4, TNFα, INF-γ, IL-6, Flt3 ligand, and IL-3. In yet other embodiments the method includes the step of contacting the dendritic cell with an antigen prior to the isolated nucleic acid.

The invention in another aspect is an isolated antigen-expressing dendritic cell population produced by the process of: exposing an isolated dendritic cell to an antigen; contacting the isolated dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the isolated nucleic acid is from about 8–80 bases in length; and allowing the isolated dendritic cell to process and express the antigen.

The isolated nucleic acid is one which contains at least one unmethylated CpG dinucleotide and which is from about 8–80 bases in length. In one embodiment the unmethylated CpG dinucleotide has a formula:

$$5'N_1X_1CGX_2N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0–25 nucleotides. In another embodiment the unmethylated CpG dinucleotide has a formula:

$$5'NX_1X_2CGX_3X_4N3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of TpT, CpT, TpC, and ApT; X_3X_4 is selected from the group consisting of GpT,GpA, ApA and ApT; N is any nucleotide and N_1+N_2 is from about 0–25 nucleotides. In a preferred embodiment N_1 and N_2 of the nucleic acid do not contain a CCGG quadmer or more than one CCG or CGG trimer. In an illustrative embodiment the isolated nucleic acid is selected from the group consisting of SEQ ID Nos. 20, 24 and 38–46. In another embodiment the isolated nucleic acid is SEQ ID NO.: 84 or 85.

In yet another embodiment the nucleotide of the isolated nucleic acid has a phosphate backbone modification, such as, for example, a phosphorothioate or phosphorodithioate modification. In one embodiment the phosphate backbone modification occurs at the 5' end of the nucleic acid. Preferably the phosphate backbone modification occurs at the first two intemucleotide linkages of the 5' end of the nucleic acid. According to another embodiment the phosphate backbone modification occurs at the 3' end of the nucleic acid. Preferably, the phosphate backbone modification occurs at the last five intemucleotide linkages of the 3' end of the nucleic acid.

According to another embodiment the isolated antigen-expressing dendritic cell is prepared by contacting the isolated dendritic cell with a cytokine selected from the group consisting of GM-CSF, IL-4, TNFα, INF-γ, IL-6, Flt3 ligand, and IL-3.

In yet another embodiment the isolated antigen-expressing dendritic cell is prepared by contacting the isolated dendritic cell with the antigen prior to the isolated nucleic acid.

The invention in another aspect is a composition, including an effective amount for synergistically activating a dendritic cell of an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length; and an effective amount for synergistically activating a dendritic cell of a cytokine selected from the group consisting of GM-CSF, IL-4, TNFα, Flt3 ligand, and IL-3. In an illustrative embodiment the cytokine is GM-CSF. In another embodiment the composition also includes an antigen, such as, for example a cancer antigen, a microbial antigen, or an allergen.

In another aspect the invention is a screening assay for identifying compounds that are effective for preventing dendritic cell maturation. The assay includes the following steps: contacting an immature dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length; exposing the dendritic cell to a putative drug; and detecting the presence or absence of a maturation marker on the dendritic cell, wherein the absence of the maturation marker indicates that the putative drug is an effective compound for preventing dendritic cell maturation. In one illustrative embodiment the maturation marker is CD83.

The invention in another aspect is a method for generating a high yield of dendritic cells. The method includes the following steps administering an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective for activating dendritic cells to a subject; allowing the isolated nucleic acid to activate dendritic cells of the subject; and isolating dendritic cells from the subject.

In another aspect the invention is a method for producing a CD40 expressing dendritic cell. The method includes the following steps: contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to produce a CD40 expressing dendritic cell.

A method for causing maturation of a dendritic cell is provided according to another aspect of the invention. The method includes the step of contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to cause maturation of the dendritic cell.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows FACS chart depicting CpG oligonucleotide promoted survival of dendritic precursor cells. Freshly isolated dendritic precursor cells were incubated for 2 days in the presence of either oligonucleotides or GMCSF (800 U/ml). Flow cytometric analysis of morphology (forward scatter, FSC; sideward scatter, SSC) showed that CpG oligonucleotides (2006: CpG phosphorothioate oligonucleotide, 1×2 μg/ml, 2080 CpG phosphodiester oligonucleotide, 3×30 μg/ml) promote survival of dendritic precursor cells, while the non CpG controls (2117: 2006 with methylated CpG; 2078: identical to 2080 but GpCs instead of CpGs) showed no positive effect on cell survival compared to the sample without oligonucleotides and GMCSF (no addition). Morphologically (FSC and SSC), viable cells were found in region A, now viable cells in region B (regions drawn in lower left dot plot).

FIG. 2 is a graph showing that the combination of CpG and GMCSF enhances viability of dendritic cells. Dendritic precursor cells were isolated from peripheral blood and incubated for 48 hours with GMCSF (800 U/ml) and oligonucleotides (2006: CpG phosphorothioate; 2117: CpGs in

2006 methylated; 2 μg/ml) as indicated. Viability was examined by flow cytometry. Data represent the mean of two independent experiments.

FIG. 3 shows FACS charts demonstrating that an increase in dendritic cell size is associated with enhanced MHC II expression. Dendritic precursor cells are incubated for 48 hours in the presence of GMCSF (800 U/ml) and oligonucleotides as indicated and examined by flow cytometry (sideward scatter, SSC). Viable cells (2500 per sample) were counted. Phosphodiester oligonucleotides (2080: CpG; 2078: non-CpG) were added at 0 hours, 12 hours and 24 hours (30 μg/ml each time point).

FIG. 4 shows FACS charts demonstrating that ICAM-1 and MHC II expression of dendritic cells in response to GMCSF and CpG. Dendritic precursor cells were incubated for 48 hours in the presence of GMCSF (800 U/ml) and 2006 (CpG phosphorothioate; 6 μg/ml). Expression of ICAM-1 (CD54) and MHC II was examined by flow cytometry (2500 viable cells are counted in each sample).

FIG. 5 is graphs depicting induction of co-stimulatory molecule expression on dendritic cells by CpG. Dendritic precursor cells were incubated for 48 hours in the presence of GMCSF (800 U/ml) and oligonucleotides (2006: CpG phosphorothioate, 6 μg/ml) as indicated. Expression of CD54 (ICAM-1) (panel A), CD86 (B7-2) (panel B) and CD40 (panel C) was quantified by flow cytometry (MFI, mean fluorescence intensity). The combination of GMCSF and 2006 shows synergy for increasing the expression of CD86 and CD40, while the effect on CD54 was additive. Results represent the mean of 5 independent experiments (CD54 and CD86) and 4 experiments (CD40). Statistical significance of the increase compared to the cell only sample is indicated by * ($p<0.05$). Statistical evaluation is performed by the unpaired t-test, error bars indicate SEM.

FIG. 6 is graphs depicting the enhancement of CD40 expression on dendritic cells is CpG specific and not induced by LPS. Dendritic precursor cells are cultured for 48 hours in the presence of GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006, CpG phosphorothioate, 6 μg/ml: 2117, methylated 2006; 2080 CpG phosphodiester, 30 μg/ml at 0 hours, 12 hours and 24 hours; 2078 GpC version of 2080). CD40 expression is examined by flow cytometry (MFI, mean fluorescence intensity). Panel A and panel B show the results of two separate sets of experiments. Panel A shows CpG specificity (methylated control oligonucleotide) for the synergy of CpG and GMCSF for induction of CD40 expression. Panel B shows that CpG is equally effective in enhancing CD40 expression as GMCSF, and that this effect is CpG specific (GpC control oligonucleotide). Panel A and B represent the mean of two independent experiments each.

FIG. 7 is graphs depicting the induction of CD54 and CD86 expression on dendritic cells is CpG specific and not induced by LPS. Dendritic precursor cells are cultured for 48 hours in the presence of GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006, CpG phosphorothioate, 2 μg/ml: 2117, methylated 2006). CD54 (panel A) and CD86 (panel B) expression is examined by flow cytometry (MFI, mean fluorescence intensity). Panel A and B represent the mean of two independent experiments (error bars indicate SEM).

FIG. 8 shows FACS charts demonstrating that CD86 expression on monocyte-derived Dendritic cells is induced by LPS but not by CpG. CD14-positive monocytes were prepared from PBMC by immunomagnetic separation and incubated in the presence of GMCSF (800 U/ml) and IL-4 (500 U/ml, Genzyme, Cambridge, Mass.). After five days (fresh medium and cytokines added every other day), cells showed the characteristic surface marker pattern of monocyte-derived dendritic cells (lineage marker negative, MHC II bright, CD1a bright, CD40 intermediate, CD54 intermediate, CD80 dim, CD86 dim) and characteristic morphology. From day 5 to day 7, LPS (1 ng/ml), TNF (1000 U/ml) or oligonucleotides in the indicated concentrations were added. CD 86 expression is measured by flow cytometry (numbers represent mean fluorescence intensity). In this series of experiments, the non-CpG phosphorothioate control oligonucleotide 2041 (5'-CTG GTC TTT CTG GTT TTT TTC TGG-3') (SEQ ID NO: 93) was used. The results are representative for 8 independent experiments, in which CpG did not stimulate monocyte-derived dendritic cells.

FIG. 9 shows FACS charts demonstrating that CpG induces maturation (CD83 expression) of dendritic cells. After 48 hours incubation with GMCSF (800 U/ml), LPS (10 ng/ml) and oligonucleotides (2006: CpG phosphorothioate; 2117 methylated 2006; 2 μg/ml), CD83 and CD86 expression on dendritic cells is determined in flow cytometry. Values (%) represent the percentage of CD83 positive (mature) cells of all viable cells. Results are representative for four independent experiments.

FIG. 10 are electron micrographs depicting CpG induction of morphologic changes in dendritic cells. Dendritic cells were incubated for 2 days in the presence of GMCSF (800 U/ml) and 2006 (2 μg/ml) (panel A), with 2006 (2 μg/ml) (panel B), with GMCSF (800 U/ml) (panel C), and with the control oligonucleotide 2117 (2 μg/ml) (panel D). Cells were fixed and processed for scanning electron microscopy according to standard procedures.

FIG. 11 are electron micrographs depicting Ultrastructural differences due to CpG Dendritic cells were incubated for 2 days in the presence of GMCSF (800 U/ml) and 2006 (2 μg/ml) (panel A) or with GMCSF (800 U/ml) (panel B) and transmission electron microscopy was performed. In the presence of CpG (panel A) multilamellar bodies (>) and multivesicular structures can be seen.

FIG. 12 are electron micrographs depicting High magnification of CpG-characteristic ultrastructural differences. Dendritic cells incubated with GMCSF (800 U/ml) and 2006 (2 μg/ml) were examined by transmission electron microscopy. Arrows point to characteristic multilamellar bodies (>) and to multivesicular structures (>>).

DETAILED DESCRIPTION OF THE INVENTION

Dendritic cells form the link between the innate and the acquired immune system by presenting antigens as well as through their expression of pattern recognition receptors which detect microbial molecules like LPS in their local environment. It has been discovered according to the invention that CpG has the unique capability to promote cell survival, differentiation, activation and maturation of dendritic cells. In fact dendritic precursor cells isolated from human blood by immunomagnetic cell sorting develop morphologic and functional characteristics of dendritic cells during a two day incubation with GM-CSF. Without GM-CSF these cells undergo apoptosis. It was discovered according to the invention that CpG was superior to GM-CSF in promoting survival and differentiation of dendritic cells (MHC II expression, cell size, granularity). As shown in the Examples below, the CpG phosphorothioate oligonucleotide 2006 (2 μg/ml) induced the expression of ICAM-1 (CD54) by 3.6-fold ($p=0.02$; $n=5$), the

co-stimulatory molecule B7-2 (CD86) by 2.4-fold (p=0.03; n=5) and CD40 by 4.1-fold (p=0.04; n=4). The combination of GM-CSF and 2006 showed a synergistic induction of CD86 and CD40, and an additive effect for CD54. Induction of CD54, CD86 and CD40 by 2006 alone was higher compared to either GM-CSF alone or GM-CSF combined with LPS. Electron microscopy revealed major ultrastructural changes of dendritic cells in response to CpG, indicating that these cells were differentiated. Additionally CpG was found to induce maturation of dendritic cells. CpG oligonucleotide 2006 was superior to GM-CSF and LPS at inducing maturation marker CD83. A synergistic maturation effect was observed when CpG oligonucleotide 2006 and GM-CSF were used together.

All effects of CpG on dendritic cells were CpG-specific as shown by control oligonucleotides with methylated CpG motifs and oligonucleotides containing GpC instead of CpG. Thus, the addition of a CpG oligonucleotide is sufficient for improving survival, differentiation, activation and maturation of human dendritic cells. Since dendritic cells form the link between the innate and the acquired immune system the ability to activate dendritic cells with CpG supports the use of CpG-based strategies for immunotherapy against disorders such as cancer and allergic or infectious diseases.

Adjuvants are nonspecific stimulators of the immune response. They are considered to be nonspecific because they only produce an immune response in the presence of an antigen. Adjuvants allow much smaller doses of antigen to be used and are essential to inducing a strong antibody response to soluble antigens (Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor, N.Y., current edition). It is shown according to the invention that CpG functions as an adjuvant by activating dendritic cells. CpG is a particularly useful adjuvant in humans because of its low toxicity. Many potent adjuvants in mice or other animals, like the Freunds complete adjuvant, cannot be used in humans due to toxicity. Additionally, as demonstrated in the examples below, CpG activates and matures human primary blood dendritic cells where other known adjuvants such as LPS fail to do so. Furthermore, CpG is known to induce a Th1 immune response which is believed to be superior to the immune response induced by alum, the adjuvant currently used in humans.

Thus the use of CpG allows the generation of mature dendritic cells from peripheral blood within two days in a well defined system. The application of CpG for this purpose is superior to GM-CSF, which is currently used for this purpose. CpG oligonucleotides have a longer half-life, are less expensive, and show a greater magnitude of immune effects. The combination of CpG and GM-CSF shows synergistic activity for the induction of co-stimulatory molecules (CD86, CD40).

The invention relates in one aspect to methods and products for activating dendritic cells for in vitro, ex vivo and in vivo purposes. It was demonstrated according to the invention that CpG oligodeoxyribonucleotides are potent activators of dendritic cells. Dendritic cells are believed to be essential for the initiation of primary immune responses in immune cells in vivo. It was discovered, according to the invention, that CpG oligodeoxyribonucleotide was capable of activating dendritic cells to initiate primary immune responses in T cells, similar to an adjuvant. It was also discovered the CpG ODN induces the production of large amounts of IL-12 in dendritic cells, indicating its propensity to augment the development of Th1 immune responses in vivo. The findings that CpG oligonucleotides were sufficient for survival, differentiation, activation, and maturation of human dendritic cells demonstrate the potent adjuvant activity of CpG and provide the basis for the use of CpG oligodeoxyribonucleotides as immunotherapeutics in the treatment of disorders such as cancer, infectious diseases, and allergy. In one aspect, the invention is a method for activating a dendritic cell by contacting the dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide, wherein the nucleic acid is from about 8–80 bases in length.

The methods and products of the invention are useful for a variety of purposes. For instance, the invention is particularly useful as an adjuvant for stimulating specific B and T cell responses to immunization. This is accomplished by contacting immature dendritic cells with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide to cause the dendritic cell to become activated and to mature. The activated dendritic cell is then incubated with resting T cells to cause activation of the T cells in order to initiate a primary immune response. In some cases the dendritic cell is also contacted with an antigen. Dendritic cells efficiently internalize process and present the soluble tumor-specific antigen to which it is exposed. The process of internalizing and presenting antigen causes rapid upregulation of the expression of major histocompatibility complex (MHC) and costimulatory molecules, the production of cytokines, and migration toward lymphatic organs where they are believed to be involved in the activation of T cells.

One specific use for the CpG nucleic acids of the invention is to activate dendritic cells for the purpose of enhancing a specific immune response against cancer antigens. The immune response may be enhanced using ex vivo or in vivo techniques. An "ex vivo" method as used herein is a method which involves isolation of an immature dendritic cell from a subject, manipulation of the cell outside of the body, and reimplantation of the manipulated cell into a subject. The ex vivo procedure may be used on autologous or heterologous cells, but is preferably used on autologous cells. In preferred embodiments, the immature dendritic cells are isolated from peripheral blood or bone marrow, but may be isolated from any source of dendritic cells. When the ex vivo procedure is performed to specifically produce dendritic cells active against a specific cancer antigen, the dendritic cells may be exposed to the cancer antigen in addition to the CpG. In other cases the dendritic cell may have already been exposed to antigen but may not be expressing the antigen on the surface efficiently. Activation will dramatically increase antigen processing. The activated dendritic cell then presents the cancer antigen on its surface. When returned to the subject, the activated dendritic cell expressing the cancer antigen activates T cells in vivo which are specific for the cancer antigen. Ex vivo manipulation of dendritic cells for the purposes of cancer immunotherapy have been described in several references in the art, including Engleman, E. G., 1997, *Cytotechnology*, 25:1; Van Schooten, W., et al., 1997, *Molecular Medicine Today*, June, 255; Steinman, R. M., 1996, *Experimental Hematology*, 24, 849; and Gluckman, J. C., 1997, *Cytokines, Cellular and Molecular Therapy*, 3:187. The ex vivo activation of dendritic cells of the invention may be performed by routine ex vivo manipulation steps known in the art, but with the use of CpG as the activator.

The dendritic cells may also be contacted with CpG using in vivo methods. In order to accomplish this, CpG is administered directly to a subject in need of immunotherapy. The CpG may be administered in combination with an antigen or may be administered alone. In some embodiments, it is preferred that the CpG be administered in the local region of the tumor.

An "antigen" as used herein is a molecule capable of provoking an immune response. Antigens include but are not limited to cells, cell extracts, polysaccharides, polysaccharide conjugates, lipids, glycolipids, carbohydrate, viruses, and viral extracts. A "cancer antigen" as used herein is a peptide associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, et al., 1994, *Cancer Research*, 54:1055, by partially purifying the antigens, by recombinant technology, or by de novo synthesis of known antigens. Cancer antigens are isolated from a tumor or cancer (e.g. tumors of the brain, lung (e.g. small cell and non-small cell), ovary, breast, prostate, colon, as well as other carcinomas and sarcomas).

The isolated dendritic cell is contacted with CpG and exposed to an antigen. Although either step may be performed first or the steps may be performed simultaneously, in one preferred embodiment the antigen is exposed to the immature dendritic cell before the cell is contacted with the CpG. It is believed that the antigen is taken up by the dendritic cell and then when the dendritic cell is contacted with the CpG, that the dendritic cell is activated to process and present the antigen. Preferably, the antigen is exposed to the cell within 48 hours of adding CpG. In a more preferred embodiment, the dendritic cell is exposed to the antigen within 24 hours of the CpG.

The antigen is exposed to the dendritic cell. As used herein, the term "exposed to" refers to either the active step of contacting the dendritic cell with an antigen in culture under conditions which promote the uptake and processing of the antigen, the passive exposure of antigen to the dendritic cell in vivo prior to isolation of the dendritic cell, or the transfection of the dendritic cell with a gene encoding the antigen, to cause processing and presentation of the antigen through the cytosolic/class I pathway. Methods for the active exposure of dendritic cells to antigen are well-known in the art. In general, purified dendritic cells are pulsed with antigen under culture conditions which promote the uptake and processing of the antigen such that the antigen will be expressed on the cell surface in association with either class I or class II MHC. Methods for transfecting dendritic cells with DNA encoding an antigen are also well-known to those of ordinary skill in the art and require only routine experimentation.

The compositions and methods of the invention are also useful for treating infectious diseases. An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. CpG is used to stimulate an antigen specific dendritic cell which can activate a T cell response against an antigen of the microorganism. The methods are accomplished in the same way as described above for the tumor except that the antigen is specific for a microorganism using a microbial antigen. A "microbial antigen" as used herein is an antigen from a microorganism and includes but is not limited to infectious virus, infectious bacteria, and infectious fungi.

Examples of infectious virus include: *Retroviridae* (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g. polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g. strains that cause gastroenteritis); Togaviridae (e.g. equine encephalitis viruses, rubella viruses); Flaviridae (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g. coronaviruses); Rhabdoviridae (e.g. vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g. ebola viruses); Paramyxoviridae (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bungaviridae (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g. reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvovirida (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus); Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

Examples of infectious bacteria include: *Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia,* Mycobacteria sps (e.g. *M. tuberculosis, M. avium, M. intracellulare, M. kansasii, M. gordonae*), *Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), Streptococcus (viridans group), *Streptococcus faecalis, Streptococcus bovis,* Streptococcus (anaerobic sps.), *Streptococcus pneumoniae, pathogenic* Campylobacter sp., Enterococcus sp., *Haemophilus influenzae, Bacillus anthracis, corynebacterium diphtheriae, corynebacterium* sp., *Erysipelothrix rhusiopathiae, Clostridium perfringens, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasturella multocida,* Bacteroides sp., *Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema Treponema pertenue,* Leptospira, and *Actinomyces israelli.*

Examples of infectious fungi include: *Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans.* Other infectious organisms (i.e., protists) include: *Plasmodium falciparum* and *Toxoplasma gondii.*

The methods of the invention are also useful for treating allergic diseases. The methods are accomplished in the same way as described above for the tumor immunotherapy and treatment of infectious diseases except that the antigen is specific for an allergen. Currently, allergic diseases are generally treated by the injection of small doses of antigen followed by subsequent increasing dosage of antigen. It is believed that this procedure produces a memory immune response to prevent further allergic reactions. These methods, however, are associated with the risk of side effects such as an allergic response. The methods of the invention avoid these problems.

An "allergen" refers to a substance (antigen) that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander, dust, fungal spores and drugs (e.g. penicillin). Examples of natural, animal and plant allergens include proteins specific to the following genuses: Canine (*Canis familiaris*); Dermatophagoides (e.g. *Dermatophagoides farinae*); Felis (*Felis domesticus*); Ambrosia (*Ambrosia artemiisfolia*; Lolium (e.g. *Lolium perenne* or *Lolium multiflorum*); Cryptomeria (*Cryptomeria*

japonica); Alternaria (*Alternaria alternata*); Alder; Alnus (*Alnus gultinoasa*); Betula (*Betula verrucosa*); Quercus (Quercus alba); Olea (*Olea europa*); Artemisia (*Artemisia vulgaris*); Plantago (e.g. Plantago lanceolata); Parietaria (e.g. Parietaria officinalis or *Parietaria judaica*); Blattella (e.g. *Blattella germanica*); Apis (e.g. *Apis multiflorum*); Cupressus (e.g. Cupressus sempervirens, *Cupressus arizonica* and *Cupressus macrocarpa*); Juniperus (e.g. *Juniperus sabinoides, Juniperus virginiana, Juniperus communis* and *Juniperus ashei*); Thuya (e.g. Thuya orientalis); Chamaecyparis (e.g. *Chamaecyparis obtusa*); Periplaneta (e.g. *Periplaneta americana*); Agropyron (e.g. *Agropyron repens*); Secale (e.g. *Secale cereale*); Triticum (e.g. *Triticum aestivum*); Dactylis (e.g. *Dactylis glomerata*); Festuca (e.g. *Festuca elatior*); Poa (e.g. *Poa pratensis* or *Poa compressa*); Avena (e.g. *Avena sativa*); Holcus (e.g. *Holcus lanatus*); Anthoxanthum (e.g. *Anthoxanthum odoratum*); Arrhenatherum (e.g. *Arrhenatherum elatius*); Agrostis (e.g. *Agrostis alba*); Phleum (e.g. *Phleum pratense*); Phalaris (e.g. *Phalaris arundinacea*); Paspalum (e.g. *Paspalum notatum*); Sorghum (e.g. *Sorghum halepensis*); and Bromus (e.g. *Bromus inermis*).

An "allergy" refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include but are not limited to eczema, allergic rhinitis or coryza, hay fever, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions. A subject having an allergic reaction is a subject that has or is at risk of developing an allergy.

"Asthma" refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

In addition to the treatment of active disorders, the methods and products of the invention can be used as a prophylactic vaccine. In this case, the CpG nucleic acid sequence is administered in vivo, preferably in the presence of an antigen or dendritic cells are prepared ex vivo and administered.

The CpG oligonucleotides of the invention are immunostimulatory molecules. An "immunostimulatory nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a dendritic cell. An immunostimulatory nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have increased immune activity.

A "nucleic acid" or "DNA" means multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). As used herein, the term refers to ribonucleotides as well as oligodeoxyribonucleotides. The term shall also include polynucleosides (i.e. a polynucleotide minus the phosphate) and any other organic base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis).

In one preferred embodiment the invention provides an isolated immunostimulatory nucleic acid sequence containing a CpG motif represented by the formula:

$$5'N_1X_1CGX_2N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0–25 nucleotides.

In another embodiment the invention provides an isolated immunostimulatory nucleic acid sequence containing a CpG motif represented by the formula:

$$5'NX_1X_2CGX_3X_4N3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of TpT, CpT, TpC, and ApT; X_3X_4 is selected from the group consisting of GpT,GpA, ApA and ApT; N is any nucleotide and N_1+N_2 is from about 0–25 nucleotides. In a preferred embodiment N_1 and N_2 of the nucleic acid do not contain a CCGG quadmer or more than one CCG or CGG trimer.

Preferably the immunostimulatory nucleic acid sequences of the invention include X_1X_2 selected from the group consisting of GpT, GpG, GpA and ApA and X_3X_4 is selected from the group consisting of TpT, CpT and GpT. For facilitating uptake into cells, CpG containing immunostimulatory nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are immunostimulatory if sufficient immunostimulatory motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic CpG motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone modification, as discussed in more detail below. For example, the modification is a phosphorothioate or phosphorodithioate modification. More particularly, the phosphate backbone modification occurs at the 5' end of the nucleic acid for example, at the first two nucleotides of the 5' end of the nucleic acid. Further, the phosphate backbone modification may occur at the 3' end of the nucleic acid for example, at the last five nucleotides of the 3' end of the nucleic acid.

Preferably the immunostimulatory CpG DNA is in the range of between 8 to 30 bases in size when it is an oligonucleotide. Alternatively, CpG dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred immunostimulatory nucleic acid molecules (e.g. for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency by stimulating an antibody (i.e. humoral response in a subject) have a relatively high stimulation index with regard to B cell, dendritic cell and/or natural killer cell responses (e.g. cytokine, proliferative, lytic or other responses).

A "nucleic acid delivery complex" shall mean a nucleic acid molecule associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g. dendritic cell surfaces and/or increased cellular uptake by target cells). Examples of nucleic acid delivery complexes include nucleic acids associated with: a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, virosome or

liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes must be sufficiently stable in vivo to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable under appropriate conditions within the cell so that the nucleic acid is released in a functional form.

"Palindromic sequence" shall mean an inverted repeat (i.e. a sequence such as ABCDEE'D'C'B'A' in which A and A' are bases capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double-stranded structures.

A "stabilized nucleic acid molecule" shall mean a nucleic acid molecule that is relatively resistant to in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter immunostimulatory nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic acid molecule has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop structure, then the nucleic acid molecule becomes stabilized and therefore exhibits more activity.

Preferred stabilized nucleic acid molecules of the instant invention have a modified backbone. It was shown according to the invention that modification of the oligonucleotide backbone provided enhanced activity of the CpG molecules of the invention when administered in vivo. CpG constructs, including at least two phosphorothioate linkages at the 5' end of the oligodeoxyribonucleotide and multiple phosphorothioate linkages at the 3' end, preferably 5, provided maximal activity and protected the oligodeoxyribonucleotide from degradation by intracellular exo- and endonucleases. Other modified oligodeoxyribonucleotides include phosphodiester modified oligodeoxyribonucleotide, combinations of phosphodiester and phosphorothioate oligodeoxyribonucleotide, methylphosphonate, methylphosphorothioate, phosphorodithioate, and combinations thereof. Each of these combinations and their particular effects on immune cells is discussed in more detail in copending PCT patent ppplication U.S. Ser. No. 08/960,774, filed on Oct. 30, 1997, the entire contents of which is hereby incorporated by reference. It is believed that these modified oligodeoxyribonucleotides may show more stimulatory activity due to enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered intracellular localization.

Both phosphorothioate and phosphodiester oligonucleotides containing CpG motifs were active in dendritic cells. However, based on the concentration needed to induce CpG specific effects, the nuclease resistant phosphorothioate backbone CpG oligonucleotides were more potent (2 μg/ml for the phosphorothioate vs. a total of 90 μg/ml for phosphodiester). In the concentration used in this study, phosphorothioate oligonucleotides without CpG motifs showed no background stimulatory activity such as that described earlier for high phosphorothioate oligonucleotide concentrations.

Other stabilized nucleic acid molecules include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Nucleic acid molecules which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Preferred vectors are those capable of autonomous replication and expression of nucleic acids to which they are linked (e.g. an episome). Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double-stranded DNA loops which, in their vector form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

A "subject" shall mean a human or vertebrate animal including a dog, cat, horse, cow, pig, sheep, goat, chicken, monkey, rat, and mouse.

The nucleic acid sequences of the invention which are useful for stimulating dendritic cells are those broadly described above. Exemplary sequences include but are not limited to those sequences shown in Table 1–7 as well as TCCATGTCGCTCCTGATGCT (SEQ ID NO: 42), TCCATGTCGTTCCTGATGCT (SEQ ID NO: 43), TCGTCGTTGTCGTTGTCGTT (SEQ ID NO: 83); TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 84), TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO: 85), GCGTGCGTTGTCGTTGTCGTT (SEQ ID NO: 86), TGTCGTTTGTCGTTTGTCGTT (SEQ ID NO: 88), TGTCGTTGTCGTTGTCGTT (SEQ ID NO: 90) TCGTCGTCGTCGTT (SEQ ID NO: 91), TCCTGTCGTTCCTTGTCGTT (SEQ ID NO: 73), TCCTGTCGTTTTTTGTCGTT (SEQ ID NO: 75), TCGTCGCTGTCTGCCCTTCTT (SEQ ID NO: 76), TCGTCGCTGTTGTCGTTTCTT (SEQ ID NO: 77), TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 84), TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO: 85) TGTCGTTGTCGTTGTCGTT (SEQ ID NO: 90), TCCATGACGTTCCTGACGTT (SEQ ID NO: 97), GTCG(T/C)T and TGTCG(T/C)T.

The stimulation index of a particular immunostimulatory CpG DNA can be tested in various immune cell assays. Preferably, the stimulation index of the immunostimulatory CgG DNA with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of ^{3}H uridine in a murine B cell culture, which has been contacted with 20 μM of ODN for 20 h at 37° C. and has been pulsed with 1 μCi of ^{3}H uridine; and harvested and counted 4 h later as described in detail in copending PCT patent application U.S. Ser. No. 08/960,774. For use in vivo, for example to treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the immunostimulatory CpG DNA be capable of effectively inducing cytokine secretion by dendritic cells.

Preferred immunostimulatory CpG nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml of IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in the Examples. Other preferred immunostimulatory CpG DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20% YAC-1 cell specific lysis or at least about 30, more preferably at least about 35 and most preferably at least about 40% 2C11 cell specific lysis.

It was found that the motifs that stimulate murine cells best differ from those that are more effective with human cells. Certain CpG oligodeoxynucleotides are poor at activating human cells (oligodeoxyribonucleotide 1707, 1708, which contain the palindrome forming sequences GACGTC and CACGTG, respectively).

The CpG oligonucleotides are used to induce survival, activation, maturation, and differentiation of dendritic cells. A dendritic cell has its ordinary meaning in the art and includes immature dendritic cells, mature dendritic cells, antigen expressing dendritic cells, non antigen expressing cells, precursor and progenitor dendritic cells. Dendritic cells are known to express different panels of cell surface molecules at different stages of development as described in more detail below. An activated dendritic cell is a dendritic cell which capable of efficiently processing antigen. Activated dendritic cells may or may not have already taken up antigen. A mature dendritic cell as used herein is a dendritic cell which expresses CD83 on the surface.

Dendritic cells useful according to the invention may be isolated from any source as long as the cell is capable of being activated by CpG to produce an active antigen expressing dendritic cell. Several in vivo sources of immature dendritic cells may be used according to the methods of the invention. For instance bone marrow dendritic cells and peripheral blood dendritic cells are both excellent sources of immature dendritic cells that are activated by CpG. Other sources may easily be determined by those of skill in the art without requiring undue experimentation, by for instance, isolating a primary source of dendritic cells and testing activation by CpG in vitro (e.g., using assays described in the Examples section). The invention also encompasses the use of any immature dendritic cells maintained in culture as a cell line as long as the cell is capable of being activated by CpG. Such cell types may be routinely identified using standard assays known in the art.

It was discovered according to the invention, that not all sources of dendritic cells that are known to be activated by cytokines to produce antigen presenting dendritic cells are capable of being activated by CpG. For instance, monocyte-derived dendritic cells are not activated by CpG. Recently, the method of monocyte-derived dendritic cells has attracted major attention because the incubation of purified CD14-positive monocytes with GM-CSF and IL-4 and subsequent maturation with conditioned medium or TNF provides large numbers of dendritic cells within one week. Romani N, et al. *J Immunol Methods* 1996; 196: 137–151. Since these cells tend to de-differentiate into macrophages in the absence of IL-4 Hausser G, et al. *Immunobiology* 1997; 197: 534–542, these dendritic cells may not resemble the physiologic situation. Although these cells are highly responsive to LPS it was discovered that monocyte-derived Dendritic cells do not respond to CpG (see Examples). It was also demonstrated that human monocytes, while highly sensitive to LPS, show a minor and delayed response to CpG.

Peripheral blood dendritic cells isolated by immunomagnetic cell sorting, which are activated by CpG, represent a more physiologic cell population of dendritic cells than monocyte derived dendritic cells. Immature dendritic cells comprise approximately 1–3% of the cells in the bone marrow and approximately 10–100 fold less in the peripheral blood. Peripheral blood cells can be collected using devices well-known in the art, e.g., Haemonetics model v. 50 apheresis device (Haemonetics, Braintree, Mass.). Red blood cells and neutrophils are removed from the blood by centrifugation. The mononuclear cells located at the interface are isolated. Methods for isolating CD4+ dendritic cells from peripheral blood have been described O'Doherty U, et al. *J Exp Med* 1993; 178: 1067–1076 and are set forth in the Examples. In the presence of GM-CSF these cells differentiate to dendritic cells with characteristic cellular processes within two days. Differentiation is accompanied by an increase in cell size, granularity and MHC class II expression, which can be easily followed using flow cytometry. Freshly isolated dendritic cells cultured in the absence of GM-CSF rapidly undergo apoptosis. Strikingly, in the presence of CpG oligonucleotides without addition of GM-CSF, both cell survival and differentiation is markedly improved compared to GM-CSF. In the presence of CpG, dendritic cells form cell clusters which when examined by ultrastructural techniques such as electron microscopy revealed characteristic dense multilamellar intracytoplasmic bodies and multi-vesicular structures, which were not present in dendritic cells incubated with GM-CSF. Scanning electron microscopy showed long veil and sheet-like processes thought to be used for intercellular interactions, and an irregular cell shape. In contrast, cells incubated with GM-CSF were round-shaped and had only minor cellular processes. In addition to promoting survival and differentiation of dendritic cells, a single addition of CpG oligonucleotide led to activation as represented by upregulation of the co-stimulatory molecules ICAM-1 (CD54), B7-2 (CD86) and CD40. The combination of CpG oligonucleotide and GM-CSF enhanced the expression of CD86 and CD40 synergistically, proving that activation is not due to CpG-induced GM-CSF.

In addition to activating dendritic cells CpG was capable of causing maturation of the dendritic cells. Maturation is assessed by the appearance of CD83, a specific marker for mature human dendritic cells. The presence of CpG alone for two days was sufficient to cause maturation of a variable percentage of the cells and the combination of GM-CSF and CpG was found to act synergistically to cause maturation of an even greater number of cells.

Each of the effects observed by culturing cells in the presence of CpG, improved survival, differentiation, activation and maturation of dendritic cells, were CpG specific since control oligonucleotides with methylated CpGs and oligonucleotides with GpC instead of CpGs were inactive. Additionally, CpG was superior to LPS in inducing both activation and maturation.

CD40-mediated activation of dendritic cells plays a key role for the induction of cytotoxic T-cells from naive T-cells. The profound changes observed in CpG-stimulated dendritic cells are similar to those seen after activation by CD40 Lanzavecchia A. Licence to kill. *Nature* 1998; 393: 413–414. Recently the central role of CD40 ligation for "superactivation" of dendritic cells has been identified. Lanzavecchia A. Licence to kill. *Nature* 1998; 393: 413–414; Schoenberger S P, et al. *Nature* 1998; 393: 480–483; Ridge J P, Di Rosa F, Matzinger P. *Nature* 1998; 393: 474–478. Bennett S R, et al. *Nature* 1998; 393: 478–480. While TNF and LPS activate dendritic cells by upregulation of co-stimulatory molecules, CD40 ligation on dendritic cells is required for the dendritic cell-dependent induction of cytotoxic T-cells from naive T-cells. CD40 ligand present on the surface of activated T helper cells provides this signal under physiologic circumstances. In addition to the data presented herein the data presented in the parent application indicate that CpG may be substitutes for CD40 ligation on dendritic cells. CD40 and CpG perform a number of parallel actions. First, CpG and CD40 both activate c-Jun NH2-terminal kinase and p38, but do not activate the extracellular receptor kinase in B cells. Second,

CD40 and CpG are each sufficient to induce proliferation of B-cells. Finally, both CD40 and CpG activate NK cells in an IL-12 dependent manner.

The ability of CpG to activate human dendritic cells differs from that of murine dendritic cells. It has also been discovered that CpG upregulates MHC class II and co-stimulatory molecules on murine Langerhans cells. In another study similar changes were described for murine bone marrow-derived Dendritic cells. Sparwasser T, et al. *Eur J Immunol* 1998; 28: 2045–2054. In both studies the efficacy of CpG to induce co-stimulatory molecules does not exceed the effects seen for LPS, to which monocytic cells are highly sensitive. Murine monocytes/macrophages are known to secrete high amounts of inflammatory cytokines in response to CpG. Since the murine cell preparation may include other myelomonocytic cells in the analysis as well a secondary indirect effect of CpG on Dendritic cells in these cell preparations may have contributed to the described activation of Dendritic cells.

It has been shown according to the invention that purified human blood dendritic cells are highly sensitive to CpG, while their response to LPS is barely detectable. The LPS concentration used in this study (10 ng/ml) is 10-fold higher than the concentration found to induce maximal cytokine secretion in human monocytes (1 ng/ml). It is important to note that murine macrophages are approximately 1000-fold less sensitive to LPS than human macrophages. In contrast to human macrophages, the low sensitivity of human blood dendritic cells to LPS and the high sensitivity to CpG is striking.

Certain Unmethylated CpG Containing Nucleic Acids Were Initially Demonstrated to Have B Cell Stimulatory Activity as Shown In Vitro and In Vivo

In the course of investigating the lymphocyte stimulatory effects of two antisense oligonucleotides specific for endogenous retroviral sequences, using protocols described in the attached Examples 1 and 2 of Co-pending parent patent application U.S. Ser. No. 08/960,774, it was surprisingly found that two out of twenty-four "controls" (including various scrambled, sense, and mismatch controls for a panel of "antisense" oligodeoxyribonucleotides) also mediated B cell activation and IgM secretion, while the other "controls" had no effect.

Two observations suggested that the mechanism of this B cell activation by the "control" oligodeoxyribonucleotides may not involve antisense effects 1) comparison of vertebrate DNA sequences listed in GenBank showed no greater homology than that seen with non-stimulatory oligodeoxyribonucleotide and 2) the two controls showed no hybridization to Northern blots with 10 μg of spleen poly A+RNA. Resynthesis of these oligodeoxyribonucleotide on a different synthesizer or extensive purification by polyacrylamide gel electrophoresis or high pressure liquid chromatography have identical stimulation, eliminating the possibility of impurity. Similar stimulation was seen using B cells from C3H/HeJ mice, eliminating the possibility that lipopolysaccharide (LPS) contamination could account for the results.

The fact that two "control" oligodeoxyribonucleotide caused B cell activation similar to that of the two "antisense" oligodeoxyribonucleotide raised the possibility that all four oligodeoxyribonucleotide were stimulating B cells through some non-antisense mechanism involving a sequence motif that was absent in all of the other nonstimulatory control oligodeoxyribonucleotide. In comparing these sequences, it was discovered that all of the four stimulatory oligodeoxyribonucleotide contained CpG dinucleotides that were in a different sequence context from the nonstimulatory control.

To determine whether the CpG motif present in the stimulatory oligodeoxyribonucleotide was responsible for the observed stimulation, over 300 oligodeoxyribonucleotide ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no CpG dinucleotides in various sequence contexts were synthesized. These oligodeoxyribonucleotide, including the two original "controls" (ODN 1 and 2) and two originally synthesized as "antisense" (ODN 3D and 3M; Krieg, A. M. *J. Immunol.* 143:2448 (1989)), were then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several oligodeoxyribonucleotides that contained CpG dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more CpG dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result from an antisense mechanism or impurity. Oligodeoxyribonucleotides caused no detectable proliferation of γδ or other T cell populations.

Mitogenic oligodeoxyribonucleotide sequences uniformly became nonstimulatory if the CpG dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the CpG dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b, 2b, 3Dd, and 3Mb). Partial methylation of CpG motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast methylation of other cytosines did not reduce oligodeoxyribonucleotide activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a CpG motif is the essential element present in oligodeoxyribonucleotide that activate B cells.

In the course of these studies, it became clear that the bases flanking the CpG dinucleotide played an important role in determining the murine B cell activation induced by an oligodeoxyribonucleotide. The optimal stimulatory motif was determined to consist of a CpG flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of oligodeoxyribonucleotide to bring the CpG motif closer to this ideal improved stimulation (e.g. Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations that disturbed the motif reduced stimulation (e.g. Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the CpG motif did not reduce stimulation (e.g. Table 1, compare ODN 1 to 1d; 3D to 3Dg; 3M to 3Me). For activation of human cells, the best flanking bases are slightly different (see Table 3).

Of those tested, oligodeoxyribonucleotides shorter than 8 bases were non-stimulatory (e.g. Table 1, ODN 4e). Among the forty-eight 8 base oligodeoxyribonucleotide tested, a highly stimulatory sequence was identified as TCAACGTT (ODN 4) which contains the self complementary "palindrome" AACGTT. In further optimizing this motif, it was found that oligodeoxyribonucleotide containing Gs at both ends showed increased stimulation, particularly if the oligodeoxyribonucleotide were rendered nuclease resistant by phosphorothioate modification of the terminal intemucleotide linkages. Oligodeoxyribonucleotide 1585 (5' GGGGTCAACGTTCAGGGGGG 3') (SEQ ID NO: 47), in which the first two and last five intemucleotide linkages are phosphorothioate modified caused an average 25.4 fold increase in mouse spleen cell proliferation compared to an average 3.2 fold increase in proliferation induced by oligodeoxyribonucleotide 1638 (5' AAAATCAACGTTGAAAAAAA 3'), which has the same sequence as ODN 1585 except that the 10 Gs at the two ends are replaced by 10 As. Th effect of the G-rich ends is cis; addition of an oligodeoxyribo-

nucleotide with poly G ends but no CpG motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated CpG were found to be more immunostimulatory.

Other octamer oligodeoxyribonucleotide containing a 6 base palindrome with a TpC dinucleotide at the 5' end were also active (e.g. Table 1, ODN 4b, 4c). Other dinucleotides at the 5' end gave reduced stimulation (e.g. ODN 4f; all sixteen possible dinucleotides were tested). The presence of a 3' dinucleotide was insufficient to compensate for the lack of a 5' dinucleotide (e.g. Table 1, ODN 4 g). Disruption of the palindrome eliminated stimulation in octamer oligodeoxyribonucleotide (e.g. Table 1, ODN 4 h), but palindromes were not required in longer oligodeoxyribonucleotide.

The kinetics of lymphocyte activation were investigated using mouse spleen cells. When the cells were pulsed at the same time as oligodeoxyribonucleotide addition and harvested just four hours later, there was already a two-fold increase in ^{3}H uridine incorporation. Stimulation peaked at 12–48 hours and then decreased. After 24 hours, no intact oligodeoxyribonucleotide were detected, perhaps accounting for the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with CpG oligodeoxyribonucleotide, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude of stimulation was concentration dependent and consistently exceeded that of LPS under optimal conditions for both. Oligonucleotides containing a nuclease resistant phosphorothioate backbone were approximately two hundred times more potent than unmodified oligonucleotides.

Cell cycle analysis was used to determine the proportion of B cells activated by CpG-oligodeoxyribonucleotide. CpG-oligodeoxyribonucleotide induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23−(marginal zone) and CD23+ (follicular) subpopulations were equally responsive to oligodeoxyribonucleotide-induced stimulation, as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that CpG-oligodeoxyribonucleotide induce essentially all B cells to enter the cell cycle.

Immunostimulatory Nucleic Acid Molecules Block Murine B Cell Apoptosis

Certain B cell lines, such as WEHI-231, are induced to undergo growth arrest and/or apoptosis in response to crosslinking of their antigen receptor by anti-IgM (Jakway, J. P., et al., "Growth regulation of the B lymphoma cell line WEHI-231 by anti-immunoglobulin, lipopolysaccharide and other bacterial products," *J. Immunol.* 137:2225 (1986); Tsubata, T., J. Wu and T. Honjo: "B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signal through CD40," *Nature*, 364:634 (1993)). WEHI-231 cells are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. oligodeoxyribonucleotide containing the CpG motif were also found to protect WEHI-23 1 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that CpG oligodeoxyribonucleotide induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, CpG nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by CpG DNA.

Method for Making Immunostimulatory Nucleic Acids

For use in the instant invention, nucleic acids can be synthesized de novo using any of a number of procedures well known in the art. For example, the B-cyanoethyl phosphoramidite method (S. L. Beaucage and M. H. Caruthers, 1981, Tet. Let. 22:1859); nucleoside H-phosphonate method (Garegg, et al., 1986, *Tet. Let.* 27:4051–4051; Froehler, et al., 1986, *Nucl. Acid. Res.* 14:5399–5407; Garegg, et al., 1986, *Tet. Let.* 27:4055–4058, Gaffney, et al., 1988), *Tet. Let.* 29:2619–2622. These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, oligonucleotides can be prepared from existing nucleic acid sequences (e.g. genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

For use in vivo, nucleic acids are preferably relatively resistant to degradation (e.g. via endo- and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid has at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made for example as described in U.S. Pat. No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Patent No. 092, 574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A., 1990, *Chem Rev.* 90:544; Goodchild, J., 1990, *Bioconjugate Chem.* 1:165). 2'-O-methyl nucleic acids with CpG motifs also cause immune activation, as do ethoxy-modified CpG nucleic acids. In fact, no backbone modifications have been found that completely abolish the CpG effect, although it is greatly reduced by replacing the C with a 5-methyl C.

For administration in vivo, nucleic acids may be associated with a molecule that results in higher affinity binding to target cell (e.g. dendritic cell) surfaces and/or increased cellular uptake by target cells to form a "nucleic acid delivery complex." Nucleic acids can be ionically, or covalently associated with appropriate molecules using techniques which are well known in the art. A variety of coupling or crosslinking agents can be used, for example protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Nucleic acids can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

Theraneutic Uses ofimmunostimulatory Nucleic Acid Molecules

Based on their immunostimulatory properties, nucleic acid molecules containing at least one unmethylated CpG dinucleotide can be used as described in detail. The nucleic acid molecules may also be used as set forth herein and in Co-pending parent patent application U.S. Ser. No. 08/960, 774.

Immunostimulatory nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better

response from the vaccine. Preferably the immunostimulatory nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally be administered in conjunction with the vaccine, which is minimally comprised of an antigen, as the conventional adjuvant may further improve the vaccination by enhancing antigen absorption.

When the vaccine is a DNA vaccine at least two components determine its efficacy. First, the antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains CpG motifs, it functions as an adjuvant for the vaccine. Thus, CpG DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of CpG DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells.

Immunostimulatory oligonucleotides and unmethylated CpG containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g. aluminum precipitates), which are inert when injected alone and are thought to work through absorbing the antigen and thereby presenting it more effectively to immune cells. Further, conventional adjuvants only work for certain antigens, only induce an antibody (humoral) immune response (Th2), and are very poor at inducing cellular immune responses (Th1). For many pathogens, the humoral response contributes little to protection, and can even be detrimental.

In addition, an immunostimulatory oligonucleotide can be administered prior to, along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness of the malignant cells to subsequent chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. CpG nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and ADCC may likewise be beneficial in cancer immunotherapy, alone or in conjunction with other treatments.

Another use of the described immunostimulatory nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated CpG nucleic acids are predominantly of a class called "Th1" which is most marked by a cellular immune response and is associated with IL-12 and IFN-γ. The other major type of immune response is termed as Th2 immune response, which is associated with more of an antibody immune response and with the production of IL-4, IL-5 and IL-10. In general, it appears that allergic diseases are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the immunostimulatory nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an immunostimulatory nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to a subject to treat or prevent an allergy.

Nucleic acids containing unmethylated CpG motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotope switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN-γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines.

As described in Co-pending parent patent application U.S. Ser. No. 08/960,774, oligonucleotides containing an unmethylated CpG motif (i.e. TCCATGACGTTCCTGACGTT; SEQ IN NO: 97), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO: 98) prevented the development of an inflammatory cellular infiltrate and eosinophilia in a murine model of asthma. Furthermore, the suppression of eosinophilic inflammation was associated with a suppression of Th2 response and induction of a Th1 response.

For use in therapy, an effective amount of an appropriate immunostimulatory nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing the oligonucleotide to be taken up by the appropriate target cells (e.g. dendritic cells). Preferred routes of administration include oral and transdermal (e.g. via a patch). Examples of other routes of administration include injection (subcutaneous, intravenous, parenteral, intraperitoneal, intrathecal, etc.). The injection can be in a bolus or a continuous infusion.

A nucleic acid alone or as a nucleic acid delivery complex can be administered in conjunction with a pharmaceutically acceptable carrier. As used herein, the phrase "pharmaceutically acceptable carrier" is intended to include substances that can be coadministered with a nucleic acid or a nucleic acid delivery complex and allows the nucleic acid to perform its indicated function. Examples of such carriers include solutions, solvents, dispersion media, delay agents, emulsions and the like. The use of such media for pharmaceutically active substances are well known in the art. Any other conventional carrier suitable for use with the nucleic acids fall within the scope of the instant invention.

The term "effective amount" of a nucleic acid molecule refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated CpG for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or fungal infection. An effective amount for use as a vaccine adjuvant could be that amount useful for boosting a subject's immune response to a vaccine. An "effective amount" for treating asthma can be that amount useful for redirecting a Th2 type of immune response that is associated with asthma to a Th1 type of response. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated CpG motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular oligonucleotide without necessitating undue experimentation.

The compositions of the invention, including activated dendritic cells, isolated CpG nucleic acid molecules, cytokines, and mixtures thereof are administered in pharmaceutically acceptable compositions. The compositions may be administered by bolus injection, continuous infusion, sustained release from implants, aerosol, or any other suitable technique known in the art.

It is also contemplated according to the methods of the invention that any compositions of the invention may also be administered in conjunction with other immune stimulating agents, such as for instance cytokines. Cytokines, include but are not limited to, IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-1 5, granulocyte-macrophage colony stimulating factor (G-MCSF), granulocyte colony stimulating factor (GCSF), interferon-y (IFN-γ), tumor necrosis factor (TNF), TGF-β, FLT-3 ligand, and CD40 ligand.

As reported herein, in response to unmethylated CpG containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFN-γ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α, TNF-β, GM-CSF, RANTES, and probably others. The increased IL-6 expression was found to occur in B cells, CD4+T cells, monocytic cells, as well as dendritic cells.

FLT3 ligand is a class of compounds described in EP0627487A2 and W094/28391. A human FLT3 ligand cDNA was deposited with the American Tissue Type Culture Collection, Rockville, Md., and assigned accession number ATCC 69382. Interleukins have been described extensively in the art, e.g., Mosley, et al., 1989, *Cell*, 59:335, Idzerda, et al., 1990, *J. Exp. Med*, 171:861. GM-CSF is commercially available as Sargramostion. Leukine (Immunex).

Systemic administration of CpG alone in some embodiments is useful for immunotherapy against antigens. Alternative agents like GM-CSF have a shorter half life, although their synergistic effects with CpG will likely make this combination useful. On the other hand, some activators of dendritic cells like LPS or inflammatory cytokines (TNF) have dose limiting toxicity, which makes their systemic use for this purpose not practical. The present study provides the functional rationale and methods for the use of CpG for dendritic cell-based immunotherapeutic strategies against cancer and for its use as an adjuvant in humans.

Systemically administered CpG oligonucleotides enhances the availability of immature and mature dendritic cells in the blood and in tissues.

The invention is also useful for in vitro screening assays. For instance, immature dendritic cells may be used in vitro to identify other CpG specific motifs which are useful for activating or causing maturation of dendritic cells. These motifs may then be used in vivo or ex vivo for activating dendritic cells. Additionally, the same type of assay may be used to identify cytokines or other immunostimulatory molecules which may have synergistic adjuvant effects when combined with isolated CpG nucleic acid sequences of the invention.

Another assay useful according to the invention is an assay for identifying compounds which inhibit dendritic cell activation or maturation. The assay would involve the addition of a putative drug to a immature dendritic cell which is activated by CpG. If the putative drug prevents activation, then it may be a compound which is therapeutically capable of inhibiting activation or maturation of the dendritic cell. Such compounds would be useful in methods of gene therapy when it is desirable to specifically inhibit the immune response to prevent an immune response against the therapeutic protein. For instance, when Factor VIII is delivered by gene therapy methods, it is desirable to prevent an immune response from developing against the therapeutic Factor VIII. It is also useful for preventing immune response to transplanted heterologous tissue.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example 1

Generation and Characterization of Dendritic Cells

Methods

Isolation of dendritic cells: Dendritic cells represent a small population of peripheral blood mononuclear cells (0.1 to 0.4 %). They express substantial levels of CD4, but lack the T cell molecules CD3, CD8, and T cell receptor, and other lineage markers (CD19, CD14, CD16, CD56) (O'Doherty U, et al., "Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium", *J Exp Med*, 1993; 178: 1067–1076). Using these characteristics, dendritic cells can be separated by high gradient immunomagnetic cell sorting using the VARIOMACS technique (Miltenyi Biotec Inc., Auburn, Calif.). Peripheral blood mononuclear cells were obtained from buffy coats of healthy blood donors (Elmer L. DeGowin Blood Center, University of Iowa) by Ficoll-Paque density gradient centrifugation (Histopaque-1077, Sigma Chemical Co., St. Louis, Mo.) as described (Hartmann G, et al., "Specific suppression of human tumor necrosis factor-alpha synthesis by antisense oligodeoxynucleotides", *Antisense Nucleic Acid Drug Dev*, 1996; 6: 291–299). Cells were resuspended in phosphate buffered saline (0.5 % bovine serum albumin, 2 mM EDTA, pH 7.4) and incubated with colloidal superparamagnetic microbeads conjugated with CD3, CD14, CD16, CD19 and CD56). Thereafter cells were passed over a depletion column in a strong magnetic field. Cells in the flow through were collected, washed two times, incubated with a microbead-conjugated antibody to CD4, and passed over a positive selection column. CD4-positive cells were eluted from the column by removal of the column from the magnetic device. Eluted cells were passed over a second column to enhance purity of the preparation. By this technique we were able to isolate 6×10^5 to 2.2×10^7 dendritic cells from 2×10^8 to 5×10^8 peripheral blood mononuclear cells in a purity of 94 to 99 % (MHC class II expression, lineage marker negative). Viability was determined by trypan blue exclusion (>95 %). In light microscopy, purified cells had the appearance of medium sized lymphocytes.

Cell culture Cells were suspended in RPMI 1640 culture medium supplemented with 10 % (v/v) heat-inactivated (56° C., 1 h) FCS (HyClone, Logan, Utha), 1.5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco BRL, Grand Island, N.Y.) (complete medium). All compounds were purchased endotoxin-tested. Freshly prepared dendritic cells (final concentration 4×10^5 cells/ml) were cultured in 96-well plates in 200 μl complete medium in a 5% CO_2 humidified incubator at 37° C. for either 48 hours or 72 hours as indicated. The cell culture medium contained 800 U/ml GMCSF (1.25×10^4 U/mg; Genzyme, Cambridge, Mass.), 10 ng/ml LPS (from salmonella typhimurium, Sigma Chemical Co., St. Louis, Mo.) or oligonucleotides as indicated.

Results

Dendritic cells can be obtained in large numbers by incubation of CD14-positive monocytes with GMCSF and IL-4 for 7 days. However, upon withdrawal of IL-4 these cells lose their dendritic cell characteristics and become

CD14 positive macrophages (Hausser G, et al. "Monocyte-derived dendritic cells represent a transient stage of differentiation in the myeloid lineage", *Immunobiology*, 1997; 197: 534–542). In addition, IL-4 induces a Th2 immune response which may not be optimal for the induction of a specific cytotoxic T-cell response. Therefore, monocyte-derived dendritic cells, despite their availability in large numbers, may not be optimal for immunotherapeutic purposes. We found that monocyte-derived dendritic cells are sensitive to LPS but surprisingly are not activated by CpG motifs (FIG. 8). It is believed that the inability of monocyte-derived DC to respond to CpG might be due to the unphysiologic methods by which these cells are prepared. Consequently, the effect of CpG oligonucleotides on primary peripheral blood DC was examined.

Physiologically, DC are present in small numbers (<0.5%) in peripheral blood mononuclear cells. Blood dendritic cells can be identified by the expression of CD4 and HLA-DR surface antigens and the absence of lineage markers (B cell, T cell, NK cell and monocyte). Immunomagnetic depletion of lineage-positive cells and subsequent positive selection of CD4-positive cells allows the isolation of DC from peripheral blood. In our experiments, we obtained 0.7 to 2.4×10^6 DC from single buffy coats (2.5 to 5×10^8 mononuclear cells). The purity of the DC preparation (MHC II bright, lineage marker negative) varied from 93% to 99%. Freshly isolated dendritic cells have the appearance of medium sized lymphocytes. During a two days incubation with GMCSF, the cells gain the specific characteristics of dendritic cells. Morphologically, they enlarge and exhibit sheet like cell processes. They express low levels of the co-stimulatory molecules CD54 (ICAM-1, adhesion molecule), CD80 (B7-1), CD86 (B7-2) and CD40. Flow cytometric characteristics of dendritic cells after two days of culture with GMCSF are depicted in table 1.

Example 2

CpG Substitutes for GMCSF for DC Survival

Methods

Oligodeoxynucleotides Unmodified (phosphodiester) and modified nuclease-resistant (phosphorothioate) oligodeoxyribonucleotide were purchased from Operon Technologies (Alameda, Calif.). The optimal motif recognized by human immune cells is different from the optimal mouse motif. Based on other studies in which we tested a large number of oligonucleotides for their ability to activate human B-cells and NK-cells, we selected particularly potent oligonucleotides as examples of a family of active CpG-containing oligonucleotides for the use in the present study. The CpG oligonucleotides used were: 2006 (24-mer), 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3' (SEQ ID NO: 84), completely phosphorothioate-modified, and 2080 (20-mer), 5'-TCG TCG TTC CCC CCC CCC CC-3' (SEQ ID NO: 94), un-modified phosphodiester. The non-CpG control oligonucleotides used were: 2117 (24-mer), 5'-TQG TQG TTT TGT QGT TTT GTQ GTT-3' (SEQ ID NO: 95), Q=5 methyl cytosine, completely phosphorothioate-modified, and 2078 (20-mer), 5'-TGC TGC TTC CCC CCC CCC CC-3' (SEQ ID NO: 96), unmodified phosphodiester. Oligonucleotides were diluted in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) using pyrogen-free reagents. Phosphorothioate oligonucleotides (2006 and 2117) were added at a final concentration of either 2 μg/ml or 6 μg/ml as indicated. Based on preliminary experiments in which no effect was seen after a single addition, phosphodiester oligonucleotides were added at 0 hours, 12 hours and 24 hours at 30 μg/ml each (total addition 90 μg/ml).

Flow cytometry Flow cytometric data on 2500 viable cells per sample or 4000 total counts were acquired on a FACScan (Beckton Dickinson Immunocytometry systems, San Jose, Calif.). Spectral overlap was corrected by appropriate compensation. Fluorescence detector settings were identical in all experiments. Analysis was performed on viable dendritic cells present within a morphologic gate (FSC, SSC, >94% of cells MHC II positive and lineage marker negative). Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Inc., Stanford, Calif.).

Results

The presence of GMCSF is required for the survival of freshly isolated DC from peripheral blood. In the absence of GMCSF, DC undergo apoptosis during the first two days of cell culture. We examined the effect of CpG oligonucleotides on survival of DC in cell culture. Freshly isolated DC were incubated in the presence of GMCSF or oligonucleotides for 48 hours. Light microscopy showed the formation of cell clusters within one day for both the sample with GMCSF alone and the sample with the CpG phosphorothioate oligonucleotide 2006. While the size of the clusters was not different between these two samples, the DC incubated with 2006 displayed longer processes seen at the surface of the clusters, resembling the morphology of mature dendritic cells. This difference was distinctive between GMCSF and 2006 samples by using light microscopy. Without GMCSF or CpG, no clusters could be found but there was an increasing number of non-viable cells as revealed by trypan blue staining. Viability of DC was quantified by flow cytometry (FIG. 1). Cell survival was dramatically improved in the presence of CpG motifs. This effect was found to be CpG specific for both phosphorothioate (2006, 2117) and phosphodiester (2080, 2078) oligonucleotides, since both non-CpG control oligonucleotides (2117: methylated version of 2006; 2078: CpGs in 2080 inverted to GpCs) showed no improved survival compared to the sample with cells only. While for the nuclease resistant phosphorothioate oligonucleotides a single addition of 2 μg/ml was sufficient, the phosphodiester oligonucleotides were added repeatedly in a higher concentration (30 μg/ml at 0 hours, 12 hours ard 24 hours).

Quantification of viability (percentage of viable cells of all counted events in flow cytometry) revealed that a single addition of 2006 (2 μg/ml) to freshly prepared DC was superior to GMCSF (800 U/ml) in promoting cell survival (74.3+–5.2% vs. 57.1+–2.3%) (FIG. 2). The combination of GMCSF and 2006 further increased the number of viable cells (81.0+–6.7%). In the presence of the control oligonucleotide 2117 (2 μg/ml) cell survival was low and comparable to the sample with cells only (10.8+–5.2% and 7.4+–4.2%). These results show that CpG can substitute for GMCSF for promoting DC survival, and that the combination of both is favorable over each of them alone.

Example 3

Increased Size and Granularity of DC Induced by CpG is Associated with Enhanced Expression of MHC II

Methods

Surface antigen staining At the indicated time points, cells were harvested and surface antigen staining was performed as previously described. Monoclonal antibodies to HLA-DR (Immu-357), CD80 (MAB104) and to CD83 (HB15A) were purchased from Immunotech, Marseille, France. All other antibodies were purchased from Pharmingen, San Diego, Calif.: mABs tc CD1a (HI149), CD3 (UCHT1), CD14 (M5E2), CD19 (B43), CD40 (5C3), CD54 (HA58), CD86

(2331 (FUN-1)). FITC-labeled IgG_1,κ (MOPC-21) and PE-labeled IgG_{2b},κ (27–35) were used to control for specific staining.

Results

Flow cytometric analysis suggested that differentiation of DC is enhanced by CpG and is associated with an increase of cell size (FSC) and granularity (SSC) (FIG. 1). The surface expression of MHC II is known to be positively correlated with differentiation of DC. DC isolated from peripheral blood were cultured in the presence of GMCSF and oligonucleotides for 48 hours, stained for HLA-DR (MHC II) and examined by flow cytometry (2500 viable cells counted) (FIG. 3). In the sample with cells only or the non-CpG oligonucleotide (2078), a large immature population with low granularity (SSC) and lower MHC II expression was found (FIG. 3 region A). A small population showed high SSC and high expression of MHC II representing differentiated DC (FIG. 3, region B). The addition of either GMCSF or the CpG oligonucleotide 2080 enhanced both granularity and MHC II expression on a per cell basis (FIG. 3 left two panels). The CpG oligonucleotide 2080 showed a superior effect compared to GMCSF indicating that CpG promotes differentiation of DC in addition to an enhancement of cell survival.

Example 4

CpG Increases Co-stimulatory Molecules on DC

Methods

Detection of endotoxin The activity of LPS is standardized by the FDA using the limulus amebocyte lysate (LAL) assay (EU/ml). The lower detection limit of the LAL-assay in our hands was 0.03 EU/ml (LAL-assay Bio Whittaker, Walkersville, Md.). The LPS sample used in our studies (from salmonella typhimurium, Sigma Chemical Co., St. Louis, Mo.) had an activity of 4.35 ng/EU. No endotoxin could be detected in the oligonucleotides (<0.075 EU/mg).

Results

Differentiation of DC by the criteria of morphology and MHC II expression is not sufficient for the induction of a specific immune response by DC. Functional activation of DC requires by the expression of co-stimulatory molecules. We examined the effect of CpG on the expression of the intercellular adhesion molecule-1 (ICAM-1, CD54), and the co-stimulatory surface molecules B7-2 (CD86) and CD40. First, we were interested if an enhanced expression of MHC II on DC (differentiation) was correlated to activation reflected by CD54 expression. No positive correlation could be found confirming that differentiation is not necessarily associated with activation of DC (FIG. 4). The expression of the co-stimulatory molecules CD54 (FIG. 5, panel A), CD86 (FIG. 5, panel B) and CD40 (FIG. 5, panel C) was quantified in flow cytometry by the mean fluorescence intensity (MFI) of viable DC. In all experiments, CpG was superior to GMCSF in enhancing expression of co-stimulatory molecules. Compared to the cells only sample, the CpG oligonucleotide 2006 enhanced the expression of CD54 (25.0+−5.7 vs. 7.0+−1.8; p=0.02, n=5), CD86(3.9+−0.8 vs. 1.6+−0.3; p=0.01; n=5) and CD40 (3.5+−1.0 vs. 0.9+−0.1; p=0.04, n=4). The combination of GMCSF and 2006 showed an additive effect for CD54 (38.5+−7.9; p=0.03; n=5), and enhanced the expression of CD86 and CD40 synergistically (CD86: 7.0+−1.6; p=0.01; n=5; CD40: 8.5+−1.0; p<0.01; n=4).

Specificity was tested using 2117 (methylated version of 2006) and 2078 (GpC version of 2080). As shown in FIG. 6 for CD40, the non-CpG oligonucleotide 2117 showed no synergistic enhancement of CD40 expression when combined with GMCSF (FIG. 6 panel A). The non-CpG oligonucleotide 2078 alone did not induce CD40 compared to cells only (FIG. 6 B). Induction of CD86 (FIG. 7 panel A) and CD54 (FIG. 7 panel B) was also found to be CpG specific.

Interestingly, LPS (10 ng/ml) showed no or only slight activation of DC isolated from peripheral blood (FIG. 6 and FIG. 7). This is surprising, since a ten-fold less concentration of LPS (1 ng/ml) stimulates human CD14-positive monocytes to express CD54 and CD86, and to produce the proinflammatory cytokines TNF and IL-6. TNF synthesis of monocytes can be found for LPS concentrations as low as 10 pg/ml, and 1 ng/ml already induces the maximal response in terms of cytokine production. Monocyte-derived DC are highly sensitive to LPS but do not respond to CpG suggesting major functional differences between monocyte-derived DC and DC isolated from peripheral blood (FIG. 8).

Example 5

CpG Induces Maturation (CD83 expression) of DC

Results

Mature human DC express the specific DC marker CD83, while immature DC do not. Mature DC effectively present antigen and maintain their stimulatory capacity while migrating from peripheral tissues to lymph nodes. Maturation of DC is thought to be essential if these cells are intended to be used for therapeutic strategies where they would be activated ex vivo, pulsed with antigens, and then reinfused into a patient. We looked at simultaneous expression of CD83 and the co-stimulatory molecule CD86 on viable DC (FIG. 9). Freshly isolated DC were incubated for 3 days with GMCSF, LPS or oligonucleotides. In the absence of either GMCSF or CpG, or with the methylated control oligonucleotide 2117 (2 μg/ml), survival of cells was poor. The remaining viable cells did not express CD83 (<2%) or CD86 (FIG. 9, right dot plot, middle row). Cells incubated with GMCSF showed low expression of CD86, and only 4.1% of the cells expressed CD83 (FIG. 9, left dot plot, lower row). If LPS (10 ng/ml) is present in addition to GMCSF, the percentage of CD83 positive cells is increased to 8.6% (FIG. 9, right dot plot, lower row). In contrast, the single addition of 2006 (2 μg/ml) renders 16% of the DC CD83 positive (FIG. 9, left dot plot, middle row). The combination of GMCSF and 2006 even enhances CD83 expression synergistically (37%) (FIG. 9, left dot plot, upper row). This induction of CD83 expression was CpG specific as shown by the control oligonucleotide 2117 in combination with GMCSF (9.7%) (FIG. 9, right dot plot, upper row). Independently of the percentage of CD83 positive cells, cells positive for CD83 also expressed higher levels of CD86. The results of FIG. 9 are representative of four independent experiments.

Example 6

Ultrastructural Changes of DC in Response to CpG

Results

We examined DC by electron microscopy to detect ultrastructural differences due to CpG. In scanning electron microscopy (FIG. 10), DC cultivated with either GMCSF and CpG (FIG. 10 A) or with CpG alone (FIG. 10B) displayed a more irregular shape, longer veil processes and sheet-like projections, and more intercellular contacts than cells cultivated with GMCSF alone (FIG. 10C) or in combination with the non-CpG control oligonucleotide (FIG. 10D). Transmission electron microscopic imaging revealed striking differences between DC generated with GMCSF

combined with CpG (FIG. 11A) and GMCSF alone (FIG. 11B). DC generated in the presence of CpG showed multilamellar intracytoplasmic bodies of high density (FIG. 11A, FIG. 12, indicated by >), which are absent without CpG (FIG. 11B). In addition, CpG-generated DC showed prominent multivesicular bodies (FIG. 11A, FIG. 12, indicated by >>), and a less heterochromatin in the nucleus. The functional significance of these ultrastructural differences is unclear.

Statistical Analysis

Data were expressed as means +/–SEM. Statistical significance of differences was determined by the unpaired two-tailed Student's t-test. Differences were considered statistically significant for $p<0.05$. Statistical analyses were performed by using StatView 4.51 software (Abacus Concepts Inc., Calabasas, Calif.).

TABLE 1

ODN	Sequence (5' to 3')†
1 (SEQ ID NO:1)	GCTAGACGTTAGCGT
1a (SEQ ID NO:2)	......T.....-..
1b (SEQ ID NO:3)	......Z.....-..
1c (SEQ ID NO:4)	.....-.-.....Z..
1d (SEQ ID NO:5)	..AT..-...GAGC.
2 (SEQ ID NO:6)	ATGGAAGGTCCAGCGTTCTC
2a (SEQ ID NO:7)	..C-.CTC..G-.-.....
2b (SEQ ID NO:8)	..Z..CTC.ZG...Z.....
2c (SEQ ID NO:9)	..Z..CTC..G-.-.....
2d (SEQ ID NO:10)	..C-.CTC..G-..-..Z..
2e (SEQ ID NO:11)	.....A-.....
3D (SEQ ID NO:12)	GAGAACGCTGGACCTTCCAT
3Da (SEQ ID NO:13)	.....-..C-.....
3Db (SEQ ID NO:14)	.....-..C-.....G..
3Dc (SEQ ID NO:15)	...C.A.....
3Dd (SEQ ID NO:16)	.....Z.....
3De (SEQ ID NO:17)	.....-.....Z.....
3Df (SEQ ID NO:18)	.....-.A.....
3Dg (SEQ ID NO:19)	.....-..CC.G.ACTG..
3M (SEQ ID NO:20)	TCCATGTCGGTCCTGATGCT
3Ma (SEQ ID NO:21)	......CT.....
3Mb (SEQ ID NO:22)	.....Z.....
3Mc (SEQ ID NO:23)	.....-..Z.....
3Md (SEQ ID NO.24)	......A-.T.....
3Me (SEQ ID NO:25)	.....-.-.....C..A.
4 (SEQ ID NO:26)	TCAACGTT
4a (SEQ ID NO:27)	....GC..
4b (SEQ ID NO:28)	...GCGC.
4c (SEQ ID NO:29)	...TCGA.
4d (SEQ ID NO:30)	..TT..AA
4e (SEQ ID NO:31)	-...-.-..
4f (SEQ ID NO:32)	C...-.-..
4g (SEQ ID NO:33)	—.-.-..CT
4h (SEQ ID NO:34)	....-.-.C

TABLE 2

5a	SEQ.ID.No:3 ATGGACTCTCCAGCGTTCTC	
5b	SEQ.ID.No:36 .....AGG....A-.-.....	
5c	SEQ.ID.No:37 ..C-.....G-.-.-.....	
5d	SEQ.ID.No:38 ....AGG..C..T.....	≦10
5e	SEQ.ID.No:40 ..C-.....G..Z.....	
5f	SEQ.ID.No:39 ..Z.....ZG..Z.....	≦10
5g	SEQ.ID.No:41 ..C-.....G-.-..Z..	
	5'GCATGACGTTGAGCT3' (SEQ.ID.NO:42)	
	5'GCTAGATGTTAGCGT3' (SEQ.ID.No:43).	

TABLE 3

512	TCCATGTCGGTCCTGATGCT
SEQ ID NO:44	
1637	.....C-.....
SEQ ID NO:45	
1615	.....G-.....
SEQ ID NO:46	
1614	.....A-.....
SEQ ID NO:47	
1636	.....-.A.....
SEQ ID NO:48	
1634	.....-.C.....
SEQ ID NO:49	
1619	.....-.T.....
SEQ ID NO:50	
1618	.....A-.T.....
SEQ ID NO:51	
1639	.....AA-.T.....
SEQ ID NO:52	
1707	.....A-.TC.....
SEQ ID NO:53	
1708	.....CA-.TG.....
SEQ ID NO:54	

TABLE 4

1585	
ggGGTCAACGTTGACgggg	(SEQ ID No 55)
1629	
——gtc——	(SEQ ID No 56)
1613	
GCTAGACGTTAGTGT	(SEQ ID No 57)
1769	
——Z——	(SEQ ID No 58)
1619	
TCCATGTCGTTCCTGATGCT	(SEQ ID No 59)
1765	
——Z——	(SEQ ID No 60)

TABLE 5

ODN	Sequence (5'–3')	SEQ ID NO.
1754	ACCATGGACGATCTGTTTCCCCTC	61
1758	TCTCCCAGCGTGCGCCAT	62
1761	TACCGCGTGCGACCCTCT	63
1776	ACCATGGACGAACTGTTTCCCCTC	64
1777	ACCATGGACGAGCTGTTTCCCCTC	65
1778	ACCATGGACGACCTGTTTCCCCTC	66
1779	ACCATGGACGTACTGTTTCCCCTC	67
1780	ACCATGGACGGTCTGTTTCCCCTC	68
1781	ACCATGGACGTTCTGTTTCCCCTC	69
1823	GCATGACGTTGAGCT	70
1824	CACGTTGAGGGGCAT	71
1825	CTGCTGAGACTGGAG	72
1828	TCAGCGTGCGCC	73
1829	ATGACGTTCCTGACGTT	74
1830[2]	RANDOM SEQUENCE	75
1834	TCTCCCAGCGGGCGCAT	76
1836	TCTCCCAGCGCGCGCCAT	77
1840	TCCATGTCGTTCCTGTCGTT	78
1841	TCCATAGCGTTCCTAGCGTT	79
1842	TCGTCGCTGTCTCCGCTTCTT	80
1851	TCCTGACGTTCCTGACGTT	81

TABLE 6

ODN[1]	sequence (5'–3')	
1840	TCCATGTCGTTCCTGTCGTT	82
1960	TCCTGTCGTTCCTGTCGTT	83
1961	TCCATGTCGTTTTTGTCGTT	84
1962	TCCTGTCGTTCCTTGTCGTT	85

TABLE 6-continued

ODN[1]	sequence (5'–3')	
1963	TCCTTGTCGTTCCTGTCGTT	86
1965	TCCTGTCGTTTTTTGTCGTT	87
1966	TCGTCGCTGTCTCCGCTTCTT	88
1967	TCGTCGCTGTCTGCCCTTCTT	89
1968	TCGTCGCTGTTGTCGTTTCTT	90
1979[2]	TCCATGTXGTTCCTGTXGTT	91
1982	TCCAGGACTTCTCTCAGGTT	92
1990	TCCATGCGTGCGTGCGTTTT	93
1991	TCCATGCGTTGCGTTGCGTT	94
2002	TCCACGACGTTTTCGACGTT	95
2005	TCGTCGTTGTCGTTGTCGTT	96
2006	TCGTCGTTTTGTCGTTTTGTCGTT	97
2007	TCGTCGTTGTCGTTTTGTCGTT	98
2008	GCGTGCGTTGTCGTTGTCGTT	99
2010	GCGGCGGGCGGCGCGCGCCC	100
2012	TGTCGTTTGTCGTTTGTCGTT	101
2013	TGTCGTTGTCGTTGTCGTTGTCGTT	102
2014	TGTCGTTGTCGTTGTCGTT	103
2015	TCGTCGTCGTCGTT	104
2016	TGTCGTTGTCGTT	105
1841	TCCATAGCGTTCCTAGCGTT	106

TABLE 7

ODN[1]	sequence (5'–3')	
1962	TCCTGTCGTTCCTTGTCGTT	107
1965	TCCTGTCGTTTTTTGTCGTT	108
1967	TCGTCGCTGTCTGCCCTTCTT	109

TABLE 7-continued

ODN[1]	sequence (5'–3')	
1968	TCGTCGCTGTTGTCGTTTCTT	110
2005	TCGTCGTTGTCGTTGTCGTT	111
2006	TCGTCGTTTTGTCGTTTTGTCGTT	112
2014	TGTCGTTGTCGTTGTCGTT	113
2015	TCGTCGTCGTCGTT	114
2016	TGTCGTTGTCGTT	115
1668	TCCATGACGTTCCTGATGCT	(SEQ.ID.NO 116)
1758	TCTCCCAGCGTGCGCCAT	(SEQ.ID.NO 117)

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 99

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gcatgacgtt gagct 15

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atggaaggtc cagcgttctc 20

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atcgactctc gagcgttctc 20

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atcgactctc gagcgttctc 20

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atcgactctc gagcgttctc 20

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gagaacgctc cagcactgat 20

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tccatgctgg tcctgatgct 20

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tcttcgaa 8

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caacgtt 7

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ccaacgtt 8

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aacgttct 8

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atcgactctc gagcgttctc 20

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<223> OTHER INFORMATION: synthetic oligonucleotide

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tccatgtcgc tcctgatgct 20

<210> SEQ ID NO 43
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tccatgtcgt tcctgatgct 20

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tccatgacgt ccctgatgct 20

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tccatcacgt gcctgatgct 20

<210> SEQ ID NO 47
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<400> SEQUENCE: 47

ggggtcaacg ttgaggggg 20

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<400> SEQUENCE: 48

ggggtcagtc ttgacgggg 19

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<400> SEQUENCE: 49

gctagacgtt agtgt 15

<210> SEQ ID NO 50
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<223> OTHER INFORMATION: m5c

<400> SEQUENCE: 50

gctagacctt agtgt 15

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tccatgtcgt tcctgatgct 20

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<210> SEQ ID NO 52
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<212> TYPE: DNA
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accatggacg atctgtttcc cctc 24

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tctcccagcg tgcgccat 18

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taccgcgtgc gaccctct 18

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cacgttgagg ggcat 15

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ctgctgagac tggag 15

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<400> SEQUENCE: 63

tcagcgtgcg cc 12

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<211> LENGTH: 17
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<400> SEQUENCE: 65

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<400> SEQUENCE: 66

tctcccagcg cgcgccat 18

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<400> SEQUENCE: 67

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<400> SEQUENCE: 68

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tcctgtcgtt cctgtcgtt 19

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<400> SEQUENCE: 72

tccatgtcgt ttttggcgtt 20

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<400> SEQUENCE: 73

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<210> SEQ ID NO 74
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<400> SEQUENCE: 74

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<400> SEQUENCE: 75

tcctgtcgtt ttttgtcgtt 20

<210> SEQ ID NO 76
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<400> SEQUENCE: 76

tcgtcgctgt ctgcccttct t 21

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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 77

tcgtcgctgt tgtcgtttct t 21

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<223> OTHER INFORMATION: m5c
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (17)...(17)
<223> OTHER INFORMATION: m5c

<400> SEQUENCE: 78

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<210> SEQ ID NO 79
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<212> TYPE: DNA
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<223> OTHER INFORMATION: synthetic oligonucleotide

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tccaggactt ctctcaggtt 20

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<400> SEQUENCE: 81

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<212> TYPE: DNA
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tcgtcgttgt cgttgtcgtt 20

<210> SEQ ID NO 84
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aaaatcaacg ttgaaaaaaa 20

We claim:

1. A method for activating a dendritic cell, comprising:

contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to activate a dendritic cell, wherein the method is performed ex vivo.

2. A method for activating a dendritic cell, comprising:

contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to activate the dendritic cell, wherein the dendritic cell is an isolated dendritic cell.

3. The method of claim 1, wherein the isolated nucleic acid has a formula:

$$5'N_1X_1CGX_2N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine, adenine, or thymine; N is any nucleotide and N_1+N_2 is from about 0–25 nucleotides.

4. The method of claim 2, wherein the method is performed ex vivo.

5. The method of claim 4, further comprising contacting the dendritic cell with an antigen prior to the isolated nucleic acid.

6. A method for activating a dendritic cell, comprising:

contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to activate the dendritic cell, wherein at least one nucleotide of the isolated nucleic acid has a phosphate backbone modification where in the method is peformed ex vivo.

7. The method of claim 6, wherein the phosphate backbone modification is a phosphorothioate or phosphorodithioate modification.

8. The method of claim 7, wherein the phosphate backbone modification occures at the 5' end of the nucleic acid.

9. The method of claim 8, wherein the nucleic acid backbone includes the phosphate backbone modification at the 5' intemucleotide linkages.

10. The method of claim 7, wherein the nucleic acid backbone includes the phosphate backbone modification at the 3' intemucleotide linkages.

11. The method of claim 10, wherein the phosphate backbone modification occurs at the last five internucleotide linkages of the 3' end of the nucleic acid.

12. The method of claim 1, wherein the isolated nucleic acid has a formula:

$$5'N_1X_1X_2CGX_3X_4N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of TpT, CpT, TpC, and ApT; X_3X_4 is selected from the group consisting of GpT, GpA, ApA and ApT; N is any nucleotide and N_1+N_2 is from about 0–25 nucleotides.

13. A method for activating a dendritic cell, comprising:

contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide in an amount effective to activate the dendritic cell, wherein the isolated nucleic acid is selected from the group consisting of SEQ ID Nos. 84 and 85.

14. A method for cancer immunotherapy, comprising:

administering an activated dendritic cell that expresses a specific cancer antigen to a subject having a cancer including the cancer antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to activate the dendritic cell.

15. A method for treating an infectious disease, comprising:

administering an activated dendritic cell that expresses a specific microbial antigen to a subject having an infection with a microorganism including the microbial antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to activate the dendritic cell.

16. A method for treating an allergy, comprising:

administering an activated dendritic cell that expresses a specific allergy causing antigen to a subject having an allergic reaction to the allergy causing antigen, wherein the activated dendritic cell is prepared by contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic is from about 8–80 bases in length in an amount effective to activate the dendritic cell.

17. A method for generating a high yield of dendritic cells, comprising:

administering an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective for activating dendritic cells to a subject;

allowing the isolated nucleic acid to activate dendritic cells of the subject; and

isolating dendritic cells from the subject.

18. A method for causing maturation of a dendritic cell, comprising

contacting a dendritic cell with an isolated nucleic acid containing at least one unmethylated CpG dinucleotide wherein the nucleic acid is from about 8–80 bases in length in an amount effective to cause maturation of the dendritic cell.

19. A method for activating a dendritic cell, comprising:

contacting a dendritic cell with an effective amount to activate a dendritic cell of an isolated nucleic acid containing at least one unmethylated CpG dinucleotide and an antigen.

20. The method of claim 19, wherein the dendritic cell is contacted with the antigen within 48 hours of contacting the dendritic cell with the isolated nucleic acid.

21. The method of claim 19, wherein the dendritic cell is contacted with the antigen within 24 hours of contacting the dendritic cell with the isolated nucleic acid.

* * * * *

US006239116B1

(12) **United States Patent**
Krieg et al.

(10) **Patent No.: US 6,239,116 B1**
(45) **Date of Patent: *May 29, 2001**

(54) **IMMUNOSTIMULATORY NUCLEIC ACID MOLECULES**

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(73) Assignees: **University of Iowa Research Foundation**, Iowa City, IA (US); **Coley Pharmaceutical Group, Inc.**, Wellesley, MA (US); **The United States of America as represented by the Department of Health and Human Services**, Washington, DC (US)

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(21) Appl. No.: **08/960,774**

(22) Filed: **Oct. 30, 1997**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/738,652, filed on Oct. 30, 1996.

(51) **Int. Cl.**[7] **A61P 37/06**
(52) **U.S. Cl.** **514/44**; 536/23.1
(58) **Field of Search** 514/44; 536/23.1

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(57) **ABSTRACT**

Nucleic acid sequences containing unmethylated CpG dinucleotides that modulate an immune response including stimulating a Th1 pattern of immune activation, cytokine production, NK lytic activity, and B cell proliferation are disclosed. The sequences are also useful a synthetic adjuvant.

49 Claims, 19 Drawing Sheets

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Liang et al, J. Clin. Invest. 98: 1119 (1996).*

* cited by examiner

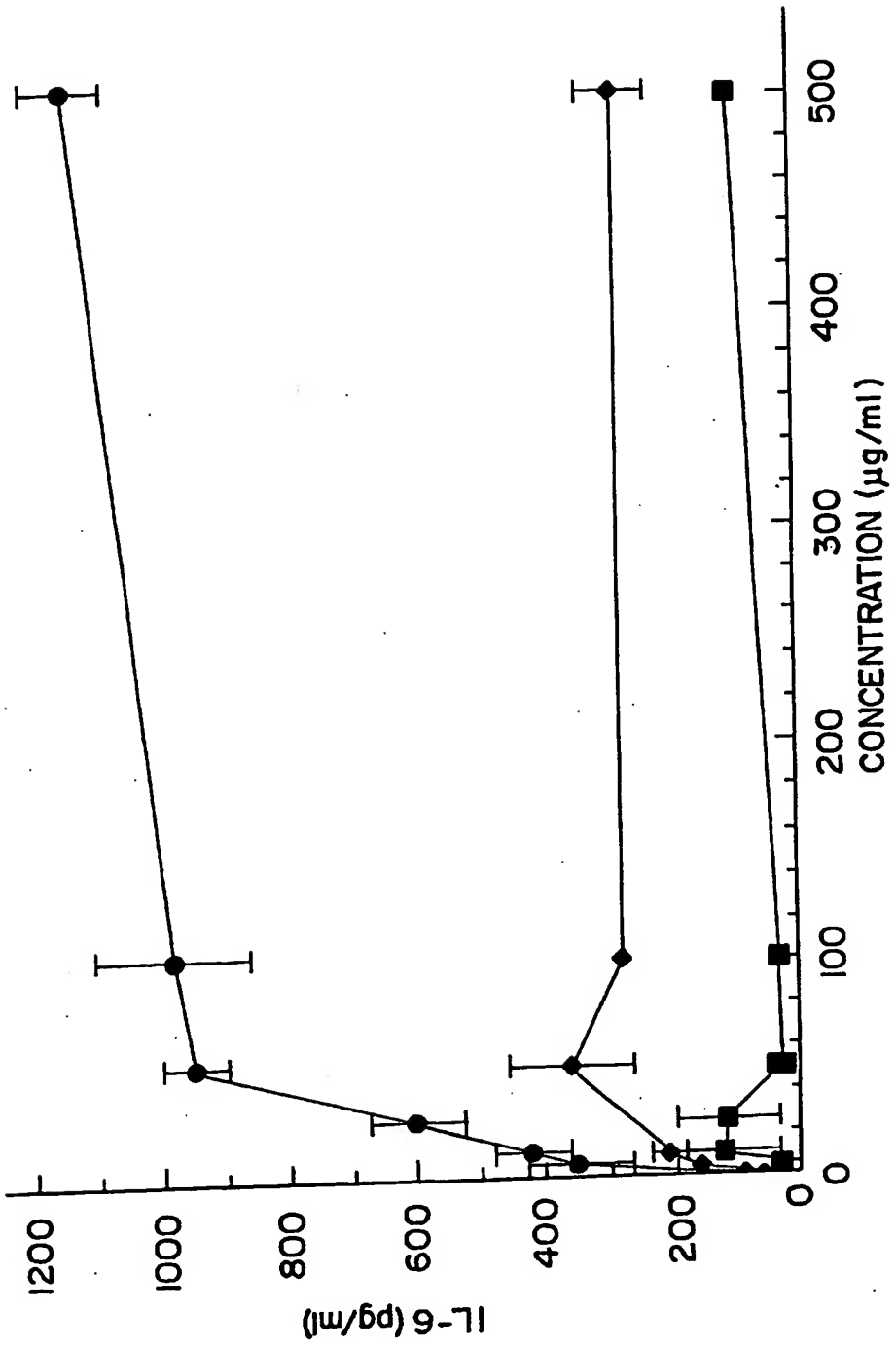
IL-6 (pg/ml)
1200
1000
800
600
400
200
0
0
100
200
300
400
500
CONCENTRATION (µg/ml)

FIG. 1A

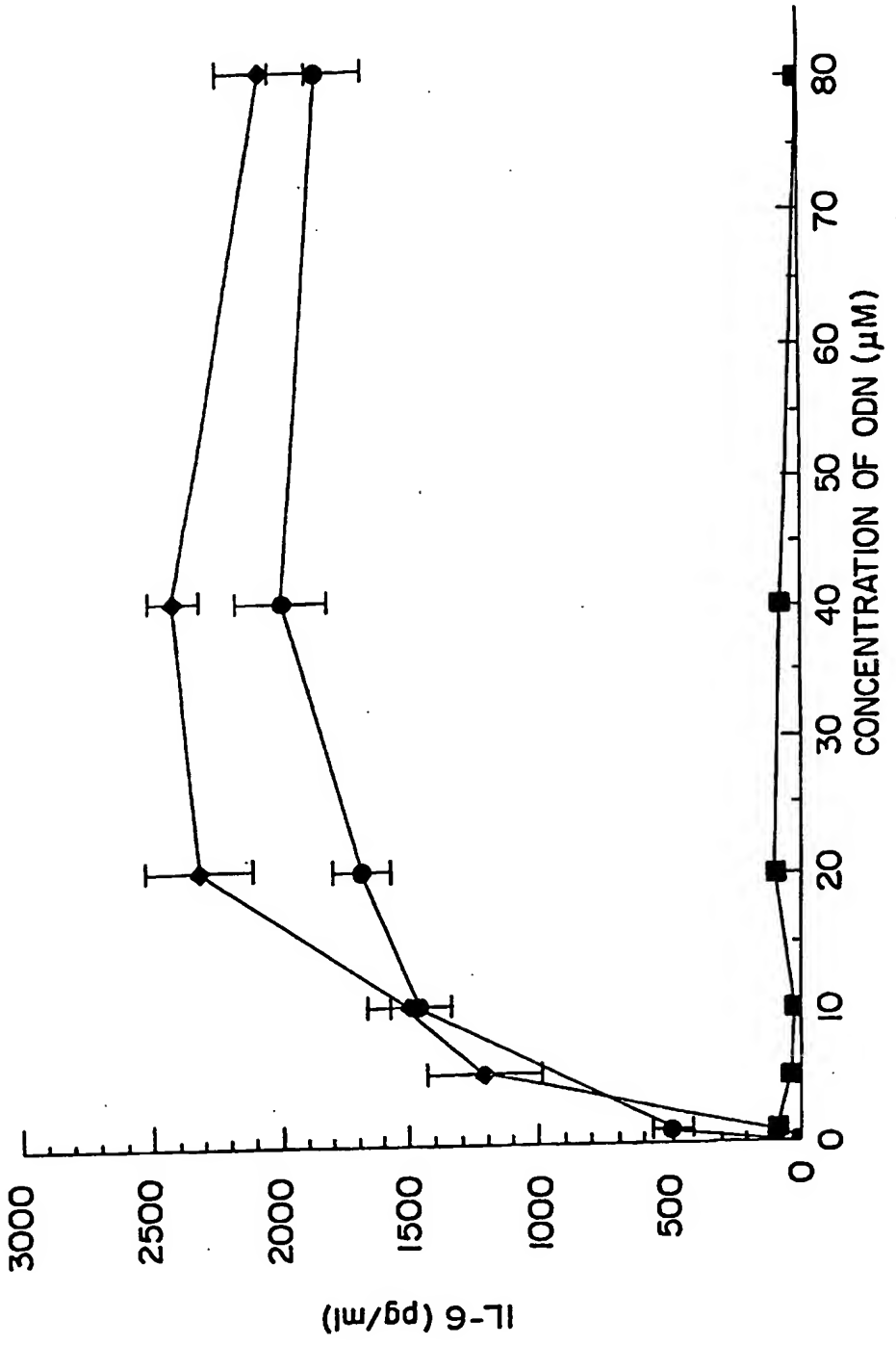
IL-6 (pg/ml)
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
80
CONCENTRATION OF ODN (μM)

FIG. 1B

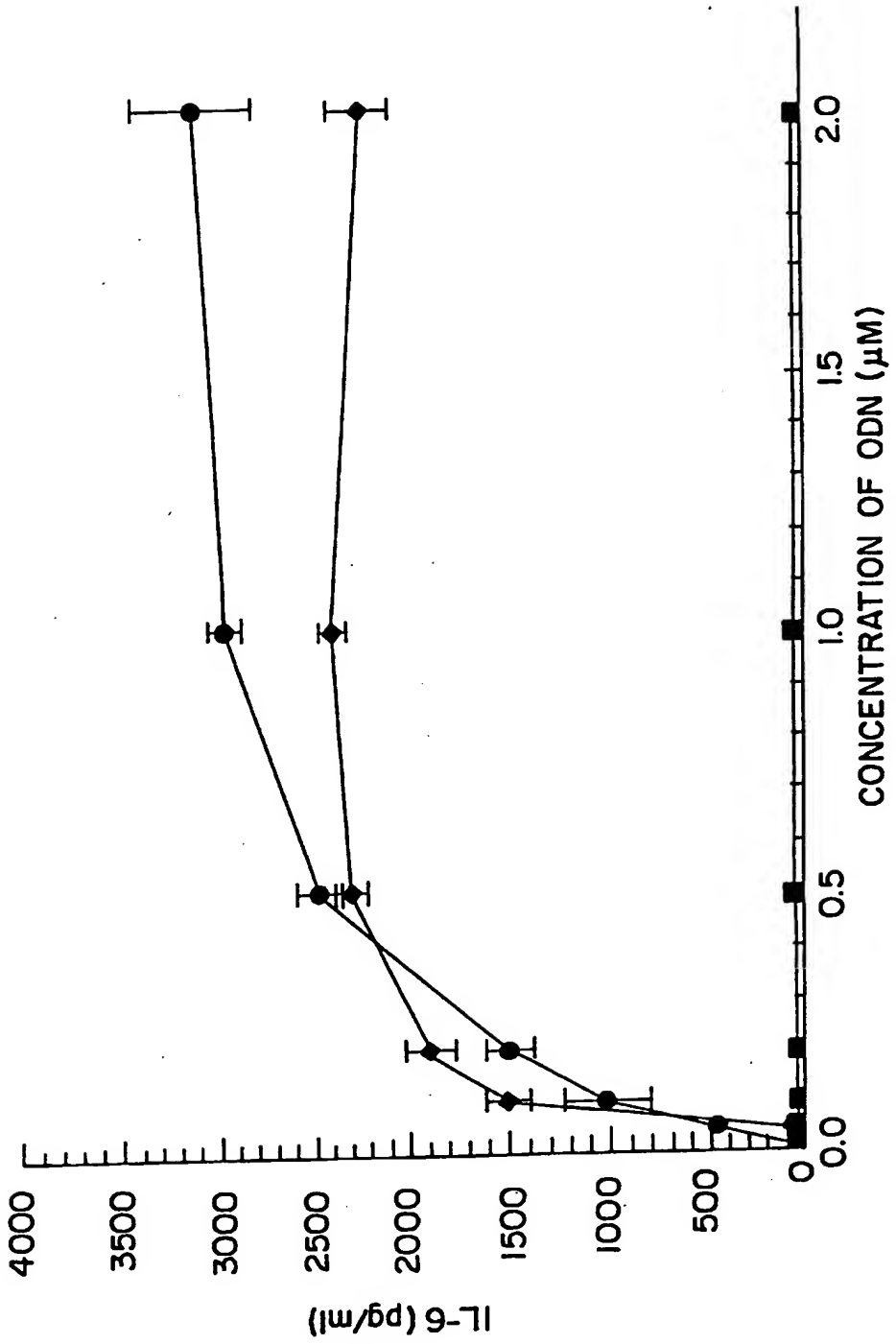
IL-6 (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
0.0
0.5
1.0
1.5
2.0
CONCENTRATION OF ODN (μM)

FIG. IC

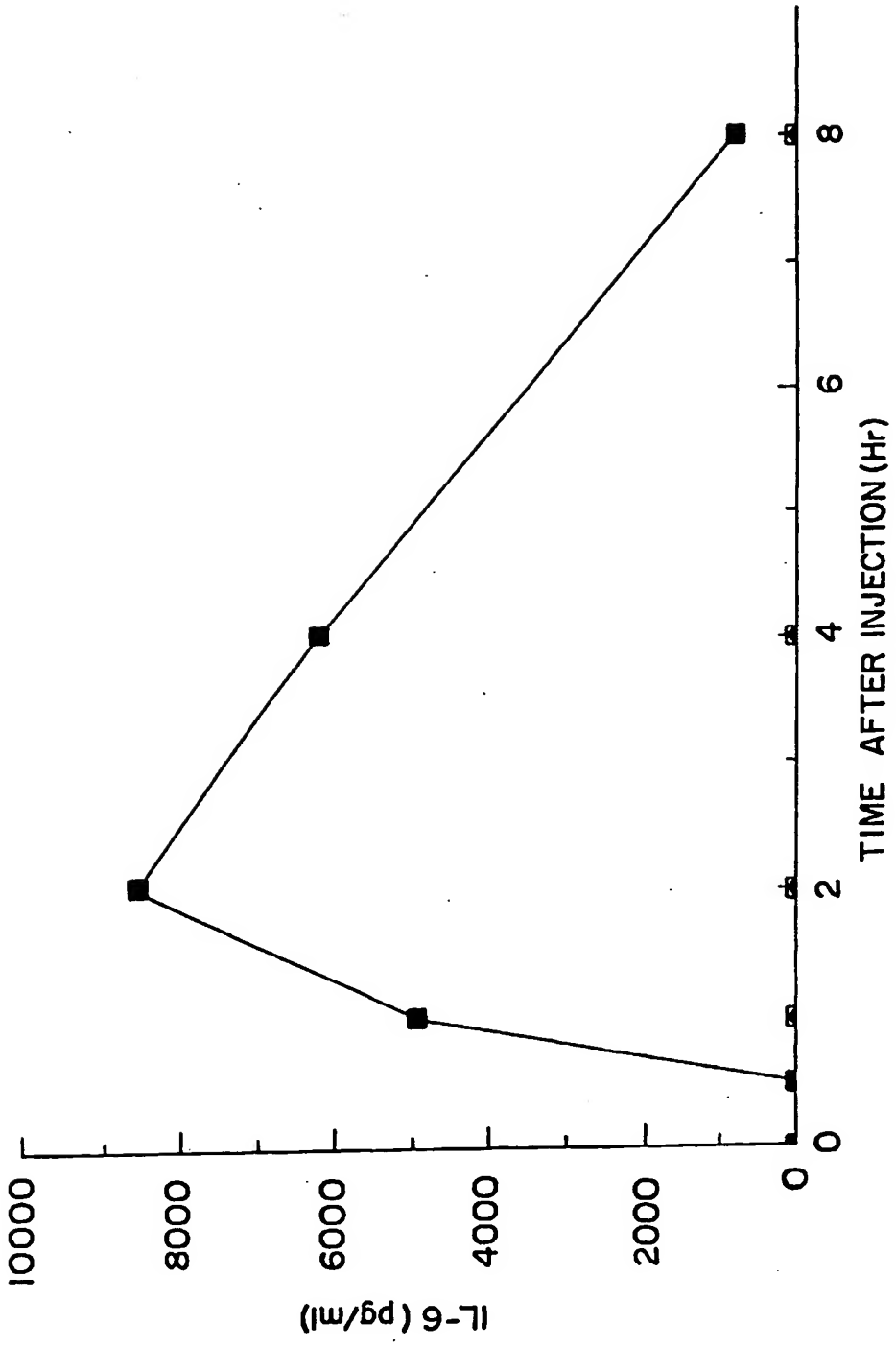
IL-6 (pg/ml)
10000
8000
6000
4000
2000
0
0
2
4
6
8
TIME AFTER INJECTION (Hr)

FIG. 2

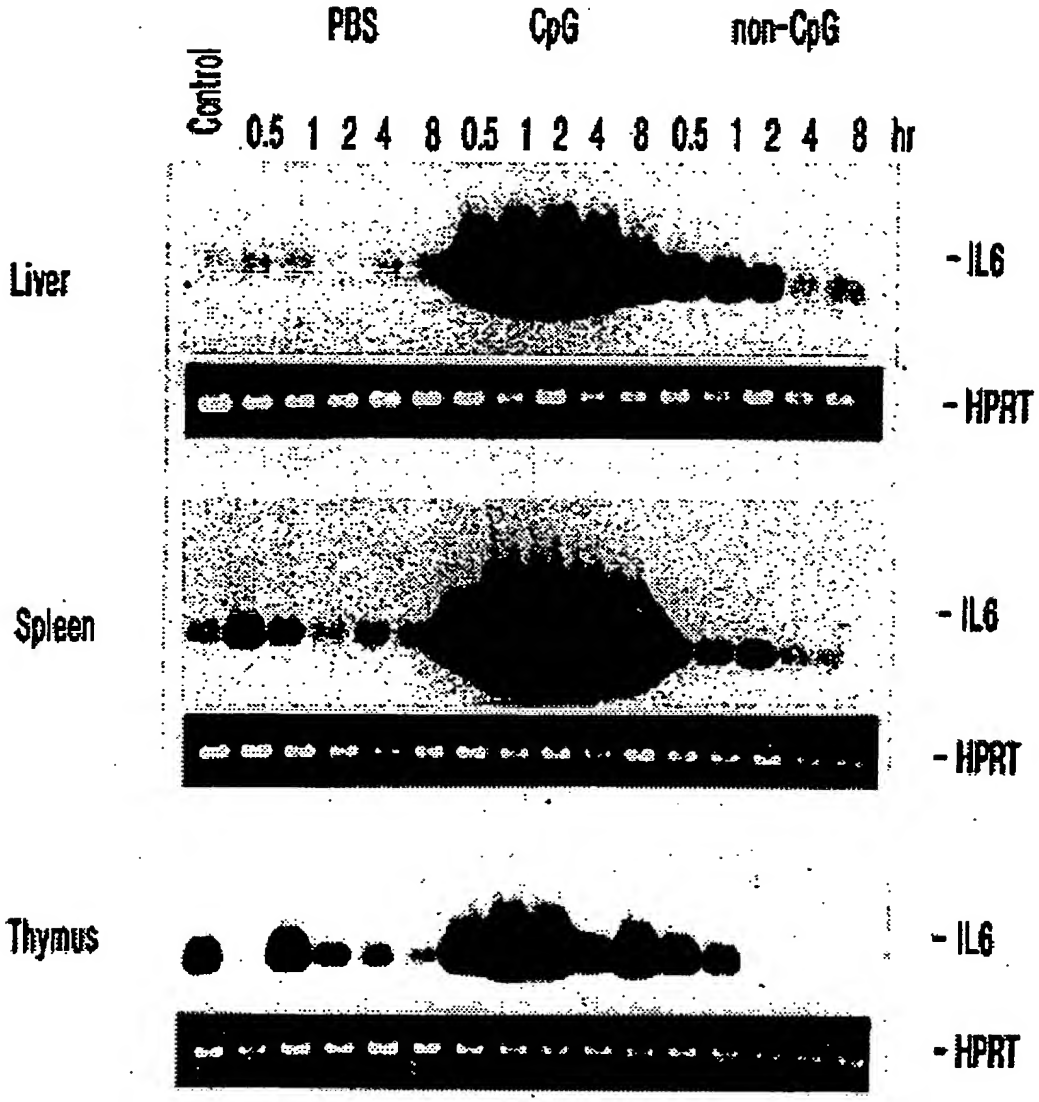
PBS
CpG
non-CpG
Control
0.5 1 2 4 8 0.5 1 2 4 8 0.5 1 2 4 8 hr
Liver
- IL6
- HPRT
Spleen
- IL6
- HPRT
Thymus
- IL6
- HPRT

FIG. 3

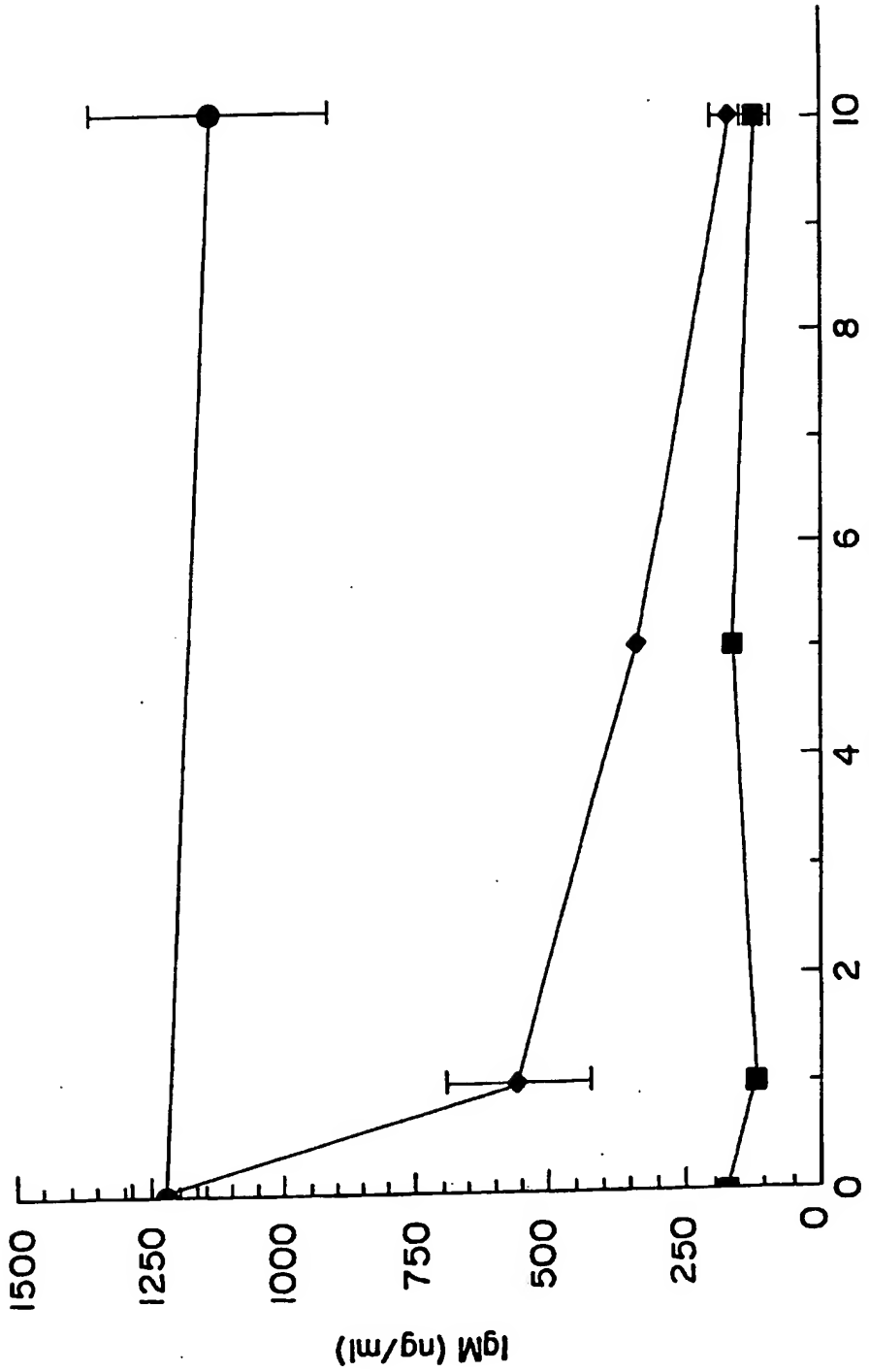
IgM (ng/ml)
1500
1250
1000
750
500
250
0
0
2
4
6
8
10
α-IL6 CONCENTRATION (μg/ml)

FIG. 4A

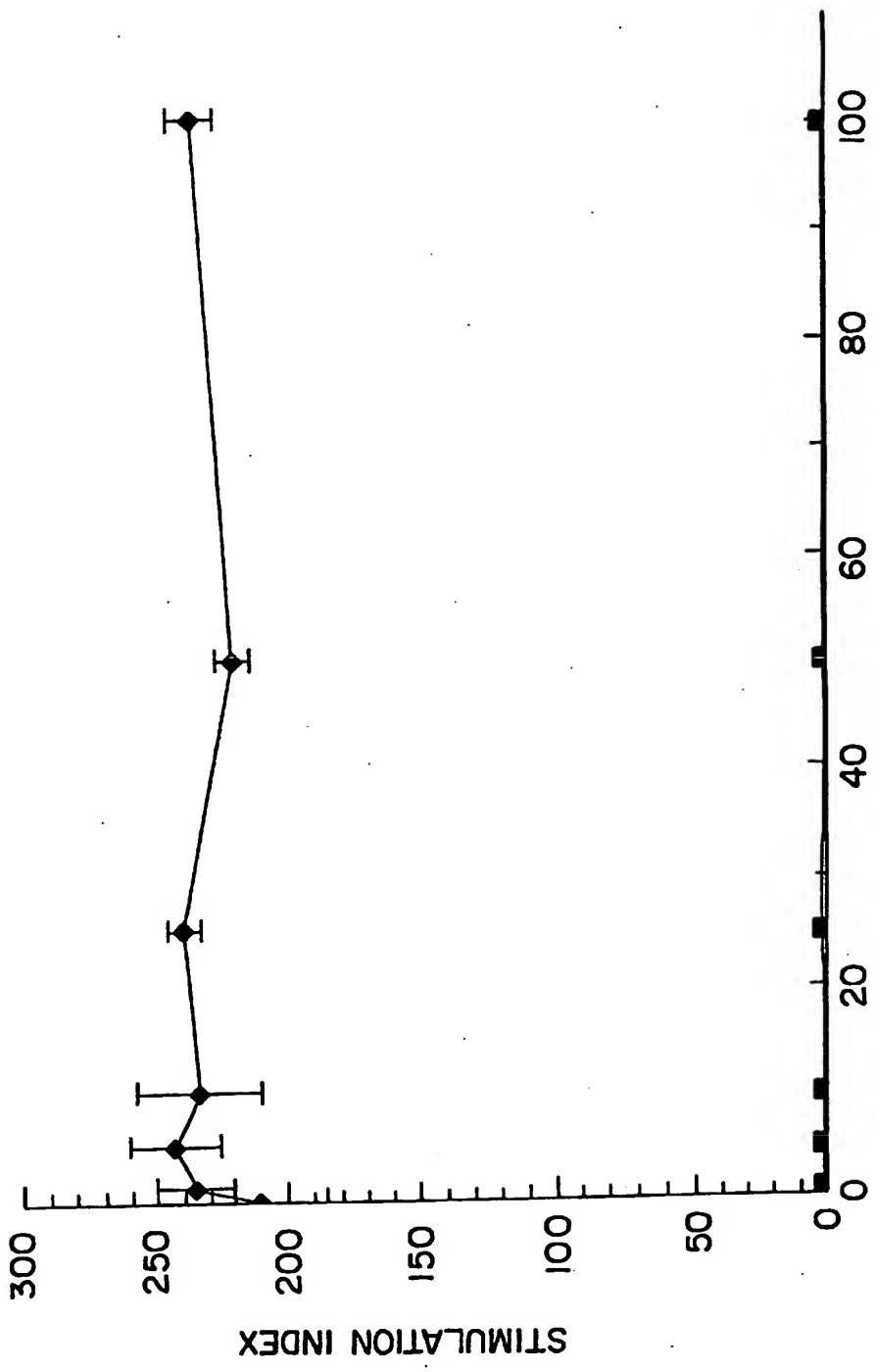
STIMULATION INDEX
300
250
200
150
100
50
0
0
20
40
60
80
100
CONCENTRATION OF ANTI-IL6 (µg/ml)

FIG. 4B

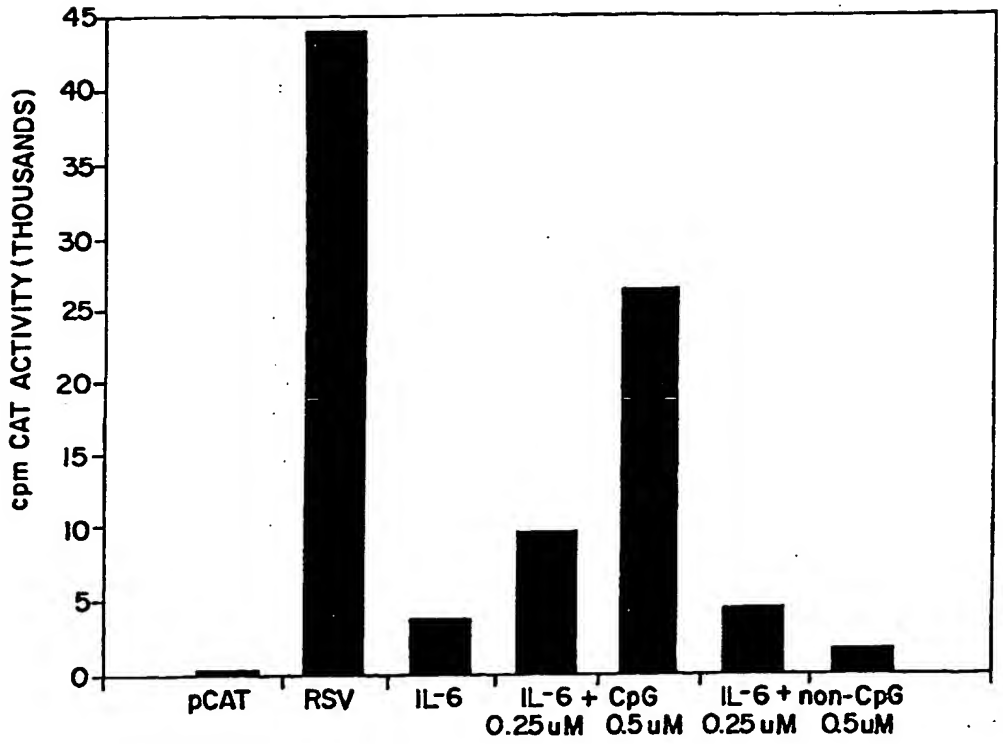
cpm CAT ACTIVITY (THOUSANDS)
45
40
35
30
25
20
15
10
5
0
pCAT
RSV
IL-6
IL-6 + CpG 0.25uM
IL-6 + CpG 0.5uM
IL-6 + non-CpG 0.25uM
IL-6 + non-CpG 0.5uM

FIG. 5

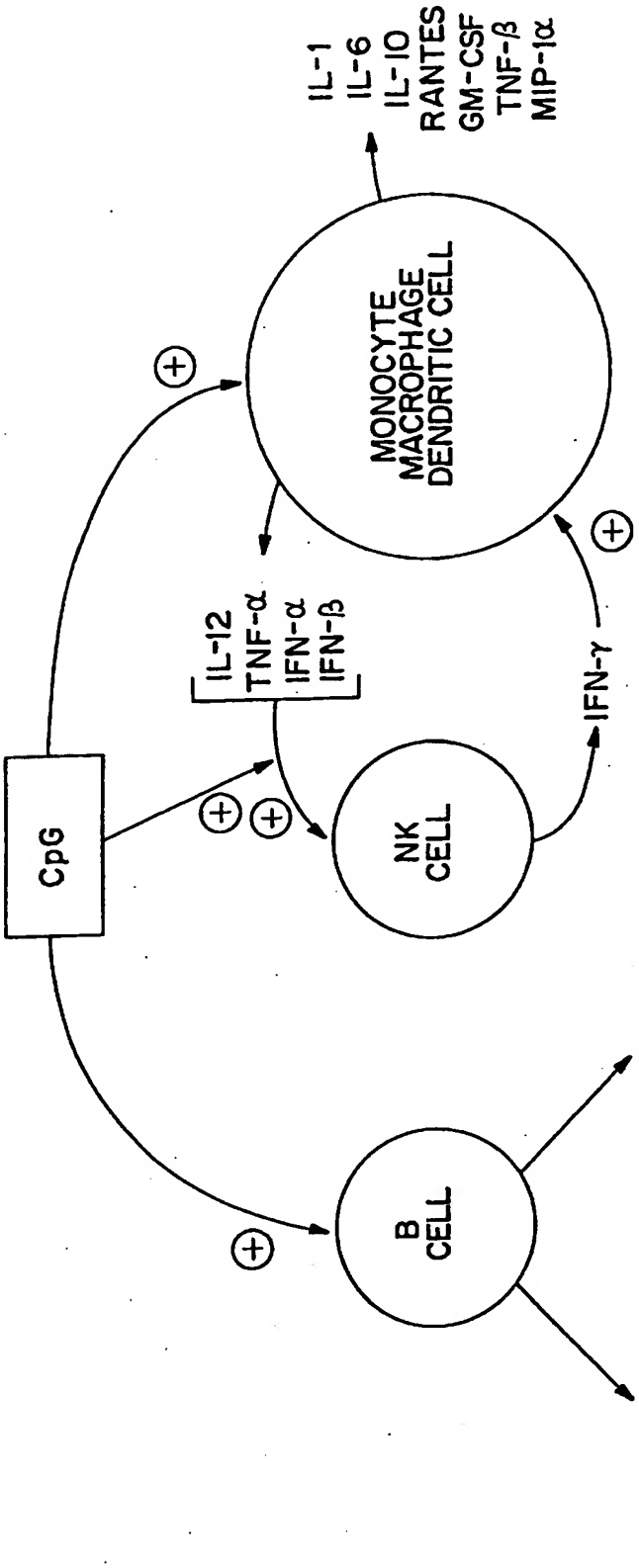
IL-1
IL-6
IL-10
RANTES
GM-CSF
TNF-β
MIP-1α
MONOCYTE
MACROPHAGE
DENDRITIC CELL
IL-12
TNF-α
IFN-α
IFN-β
IFN-γ
CpG
NK
CELL
B
CELL
PROLIFERATE
EXPRESS CLASS II MHC,
B7-1, B7-2
IL-6
IMMUNOGLOBULIN

FIG. 6

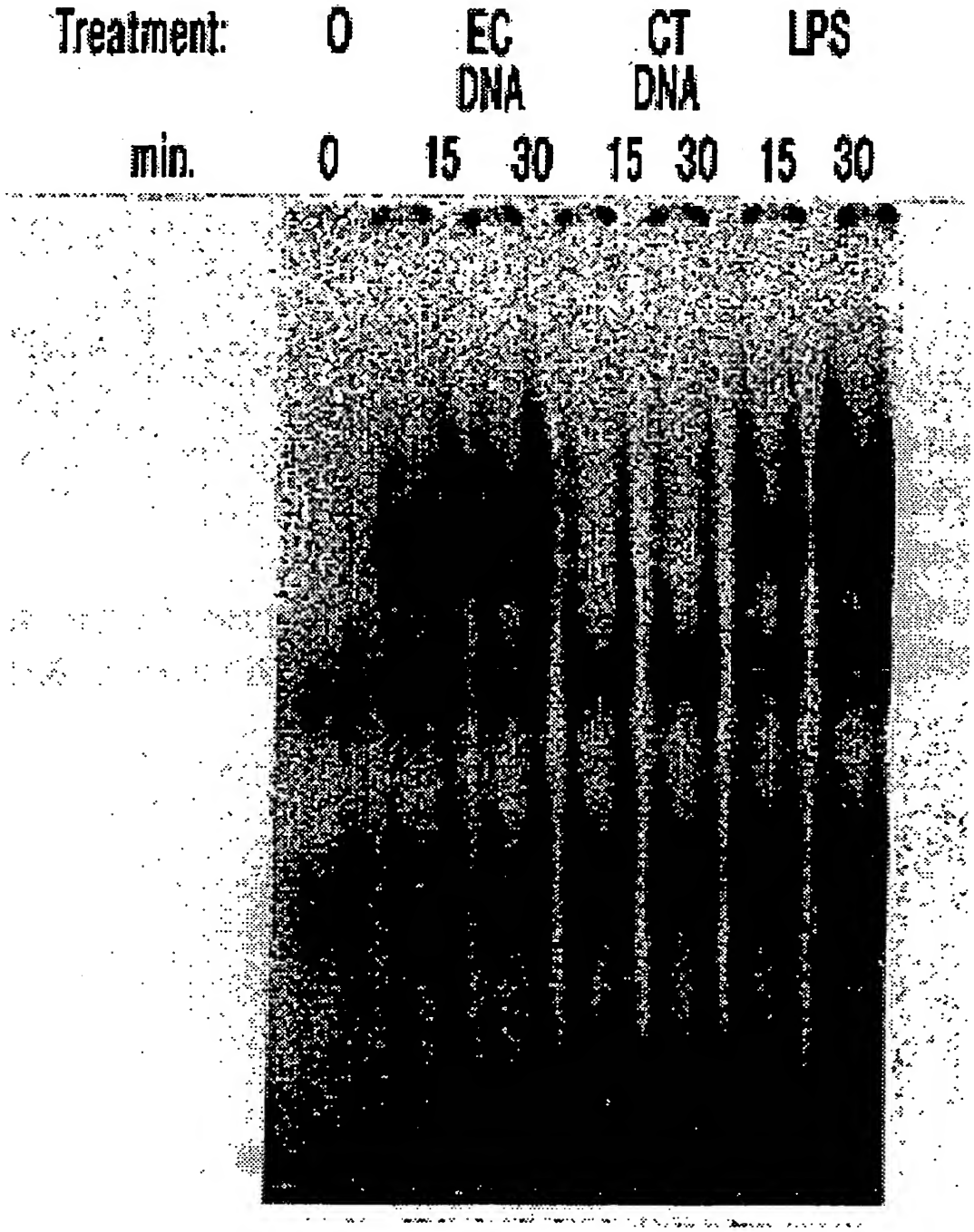

FIG. 7

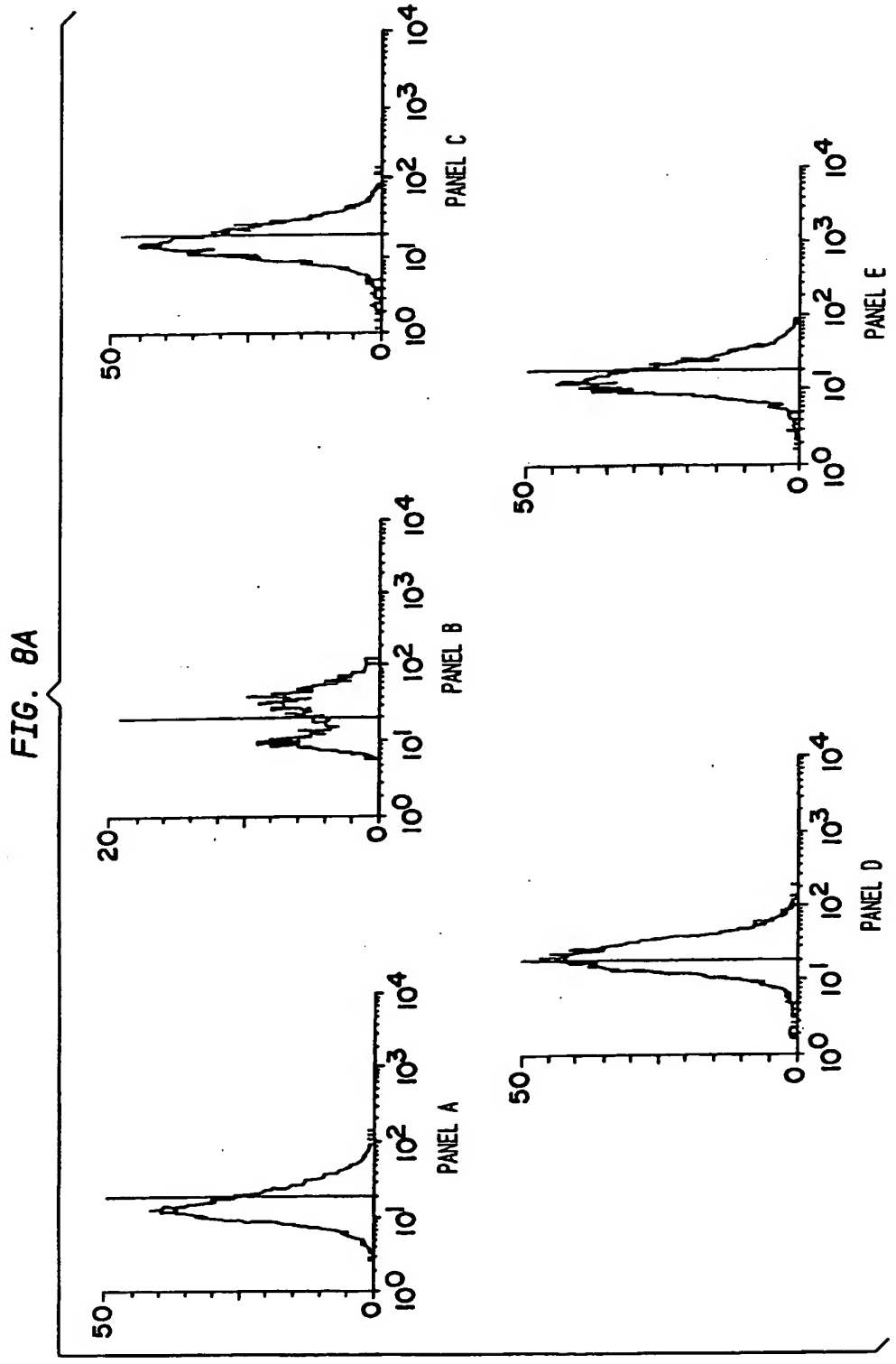
PANEL A
PANEL B
PANEL C
PANEL D
PANEL E
0
10^0
10^1
10^2
10^3
10^4
50
20

FIG. 8A

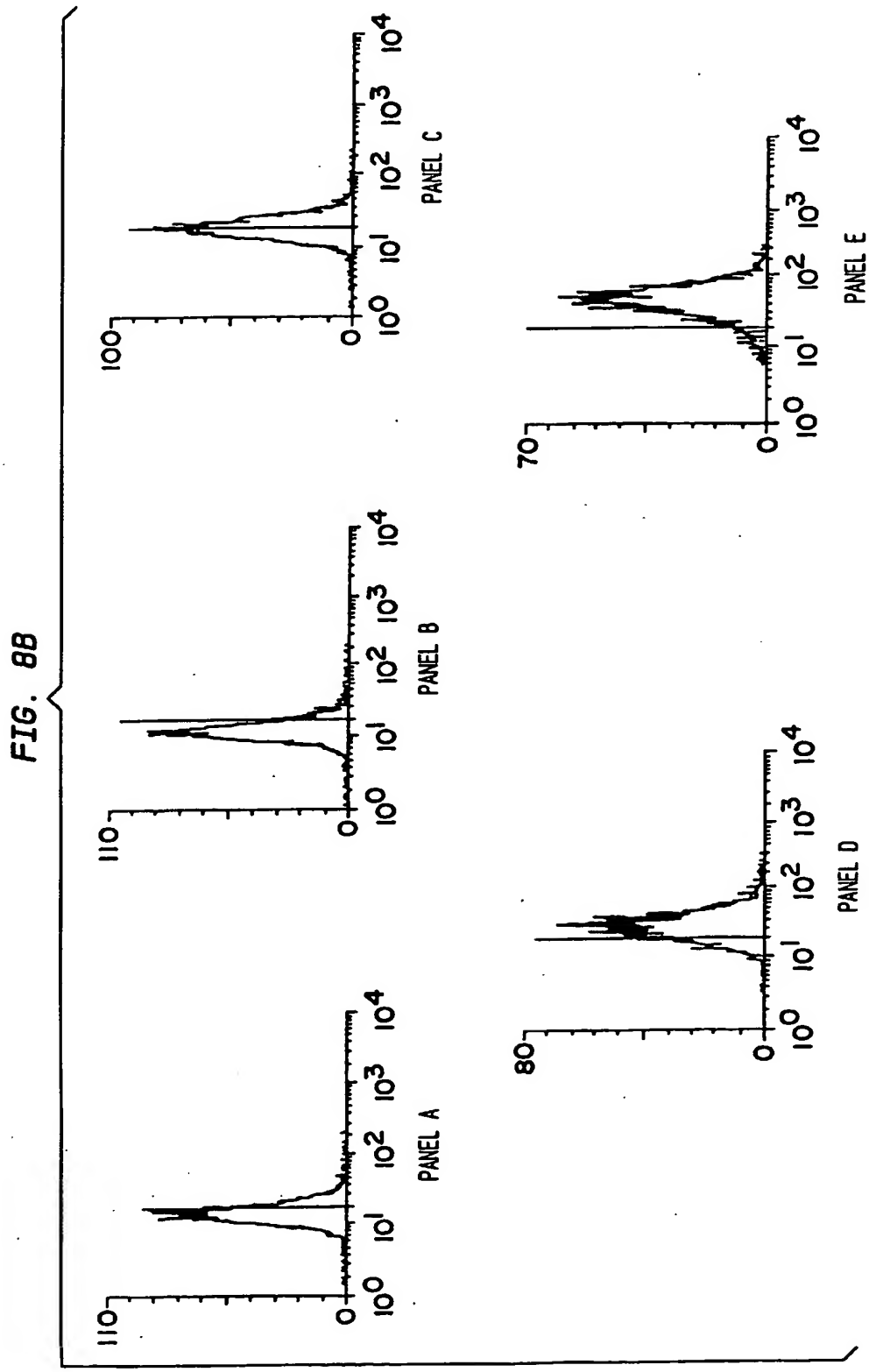
PANEL A
PANEL B
PANEL C
PANEL D
PANEL E
0
10^0
10^1
10^2
10^3
10^4
110
110
100
80
70

FIG. 8B

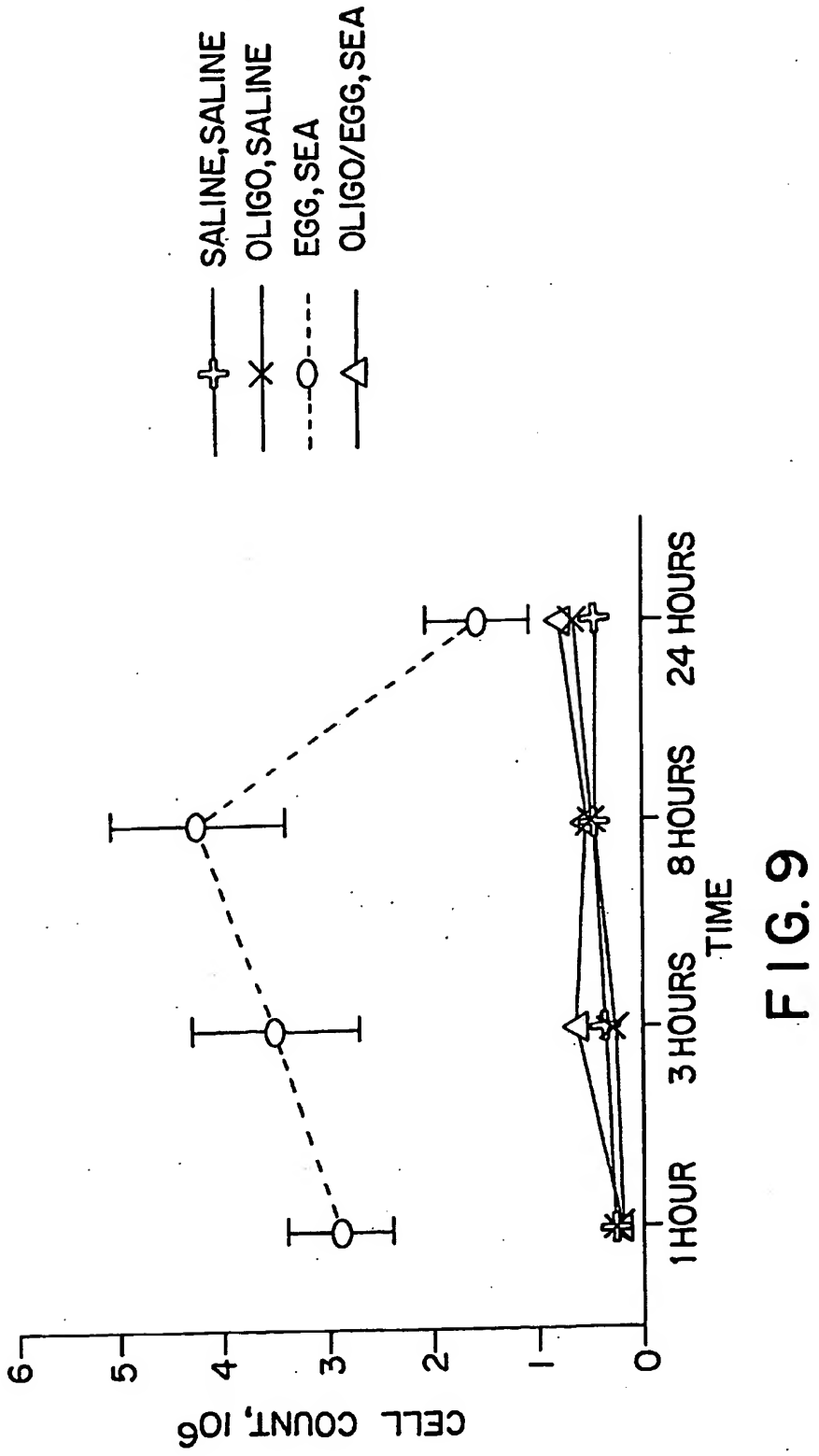
SALINE,SALINE
OLIGO,SALINE
EGG, SEA
OLIGO/EGG,SEA
CELL COUNT, 10^6
0
1
2
3
4
5
6
1 HOUR
3 HOURS
8 HOURS
24 HOURS
TIME

FIG. 9

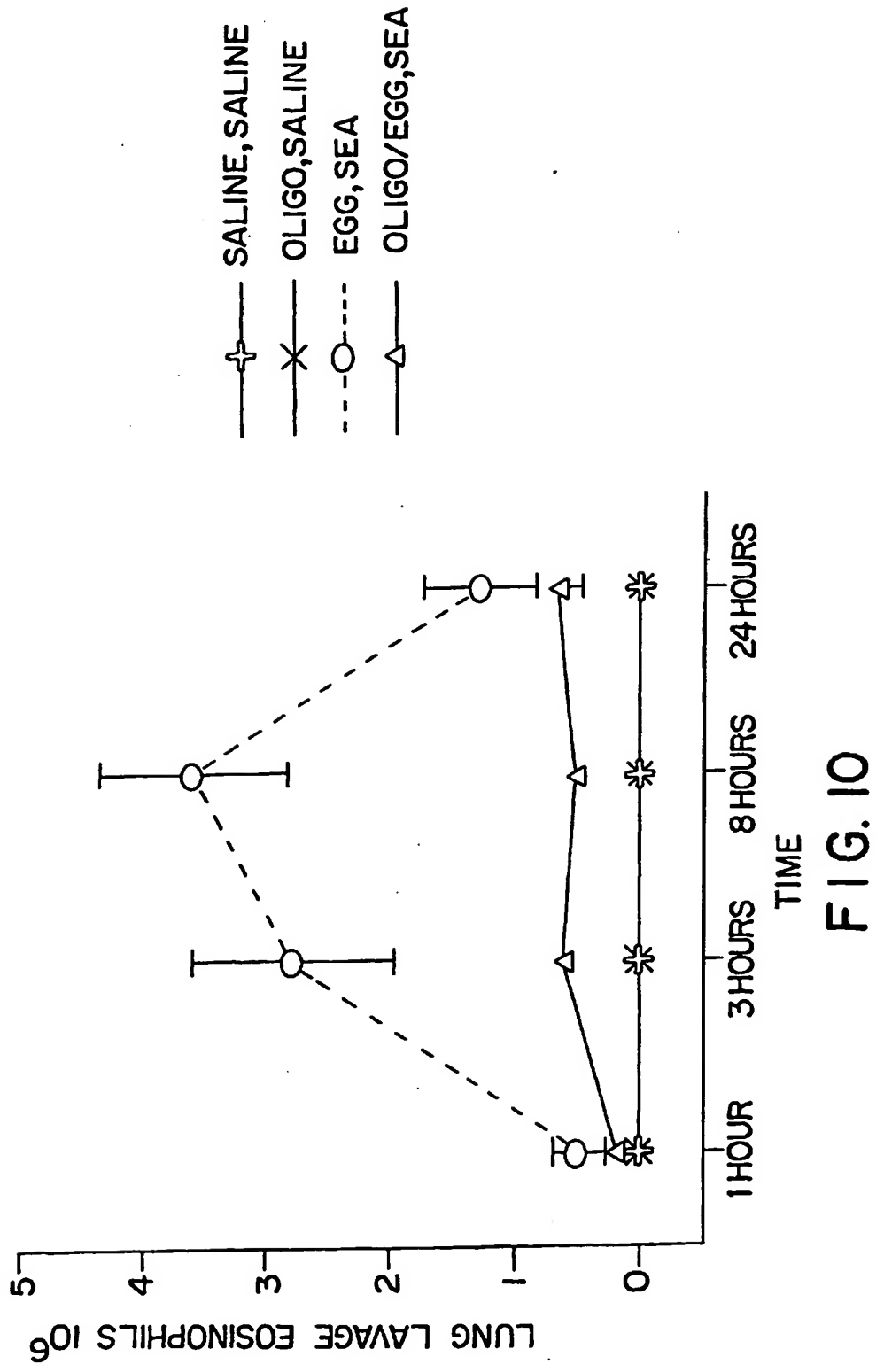
SALINE, SALINE
OLIGO, SALINE
EGG, SEA
OLIGO/EGG, SEA
LUNG LAVAGE EOSINOPHILS 10^6
0
1
2
3
4
5
1 HOUR
3 HOURS
8 HOURS
24 HOURS
TIME

FIG. 10

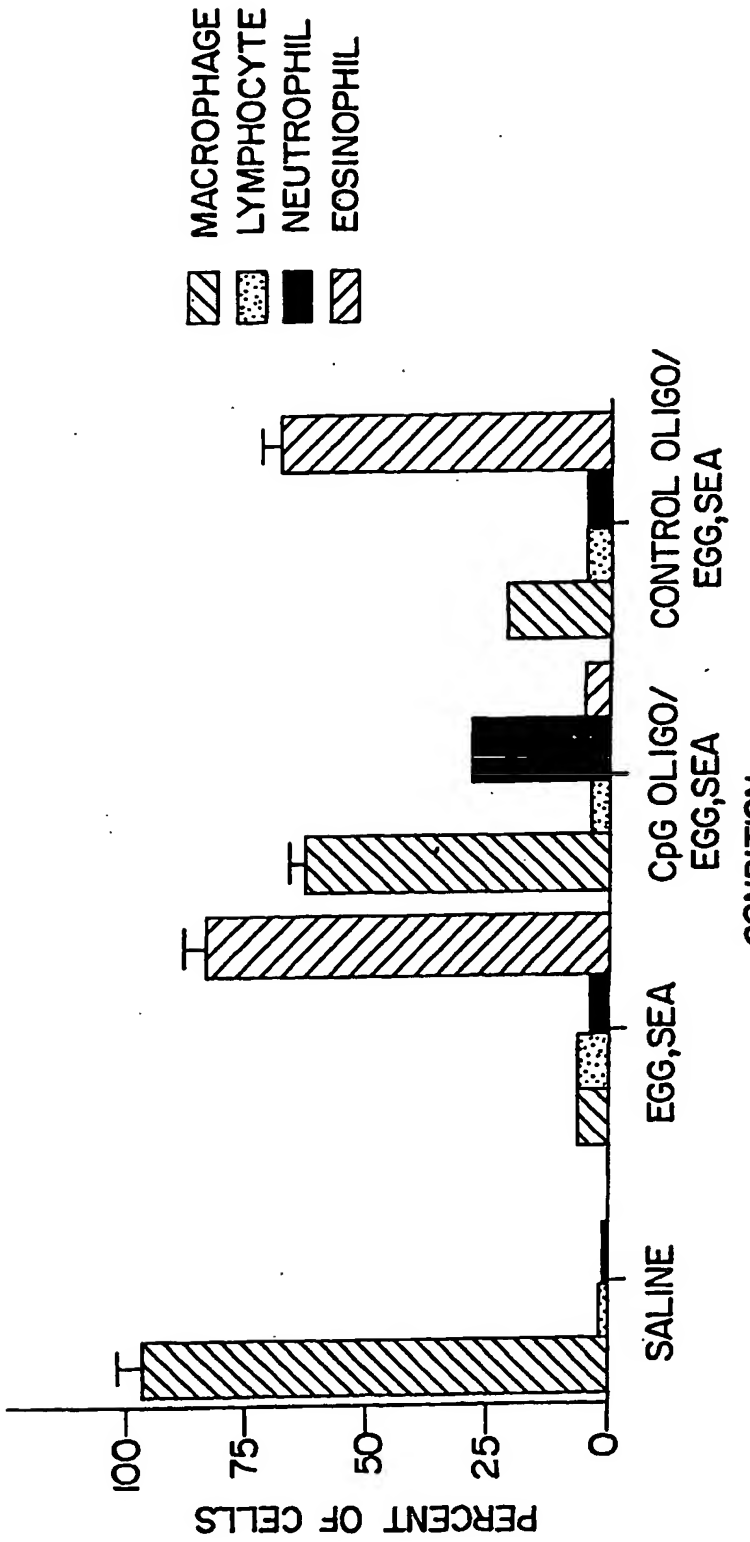
MACROPHAGE
LYMPHOCYTE
NEUTROPHIL
EOSINOPHIL
PERCENT OF CELLS
100
75
50
25
0
SALINE
EGG,SEA
CpG OLIGO/ EGG,SEA
CONTROL OLIGO/ EGG,SEA
CONDITION

FIG. 11

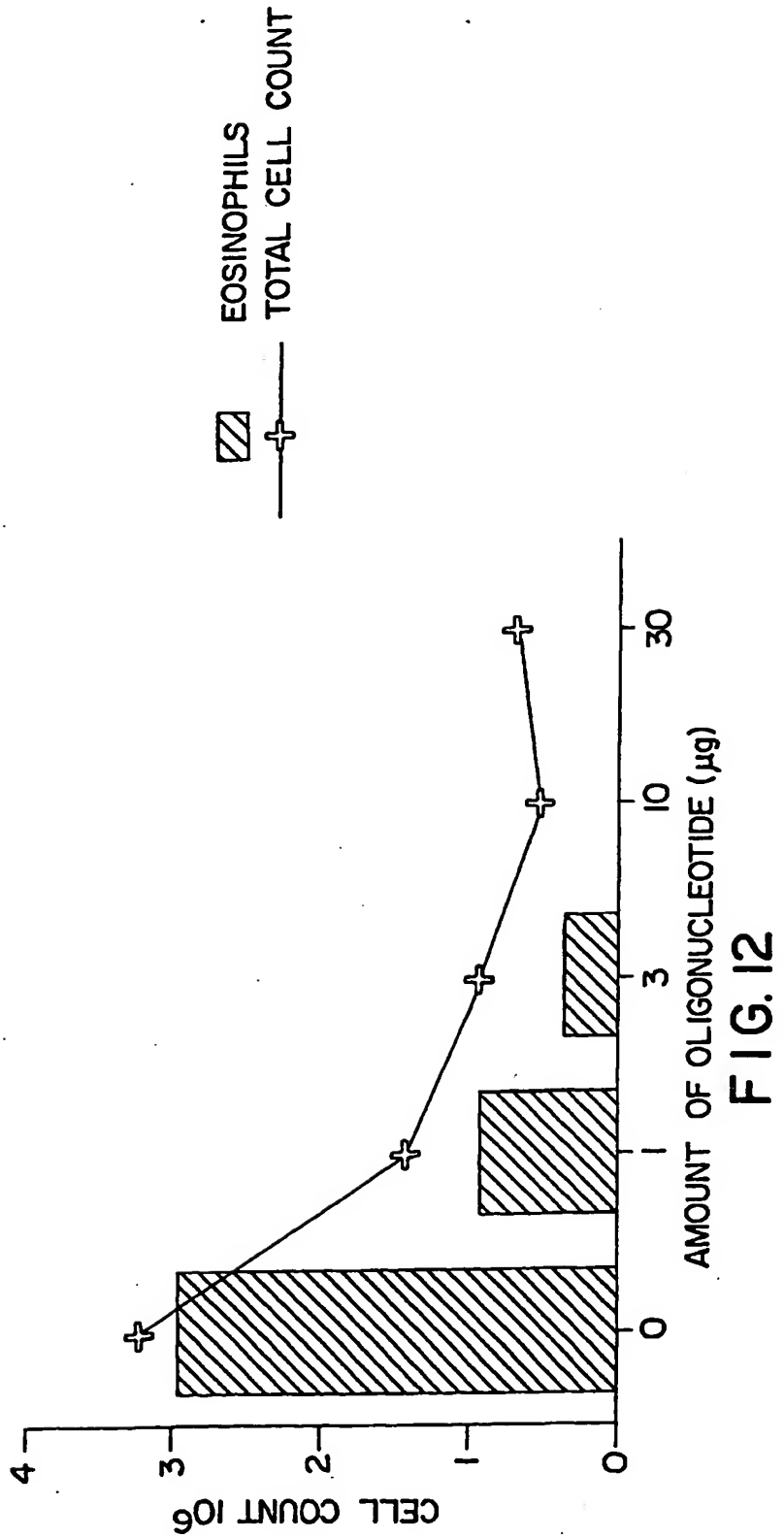
EOSINOPHILS
TOTAL CELL COUNT
CELL COUNT 10^6
0
1
2
3
4
0
1
3
10
30
AMOUNT OF OLIGONUCLEOTIDE (μg)

FIG. 12

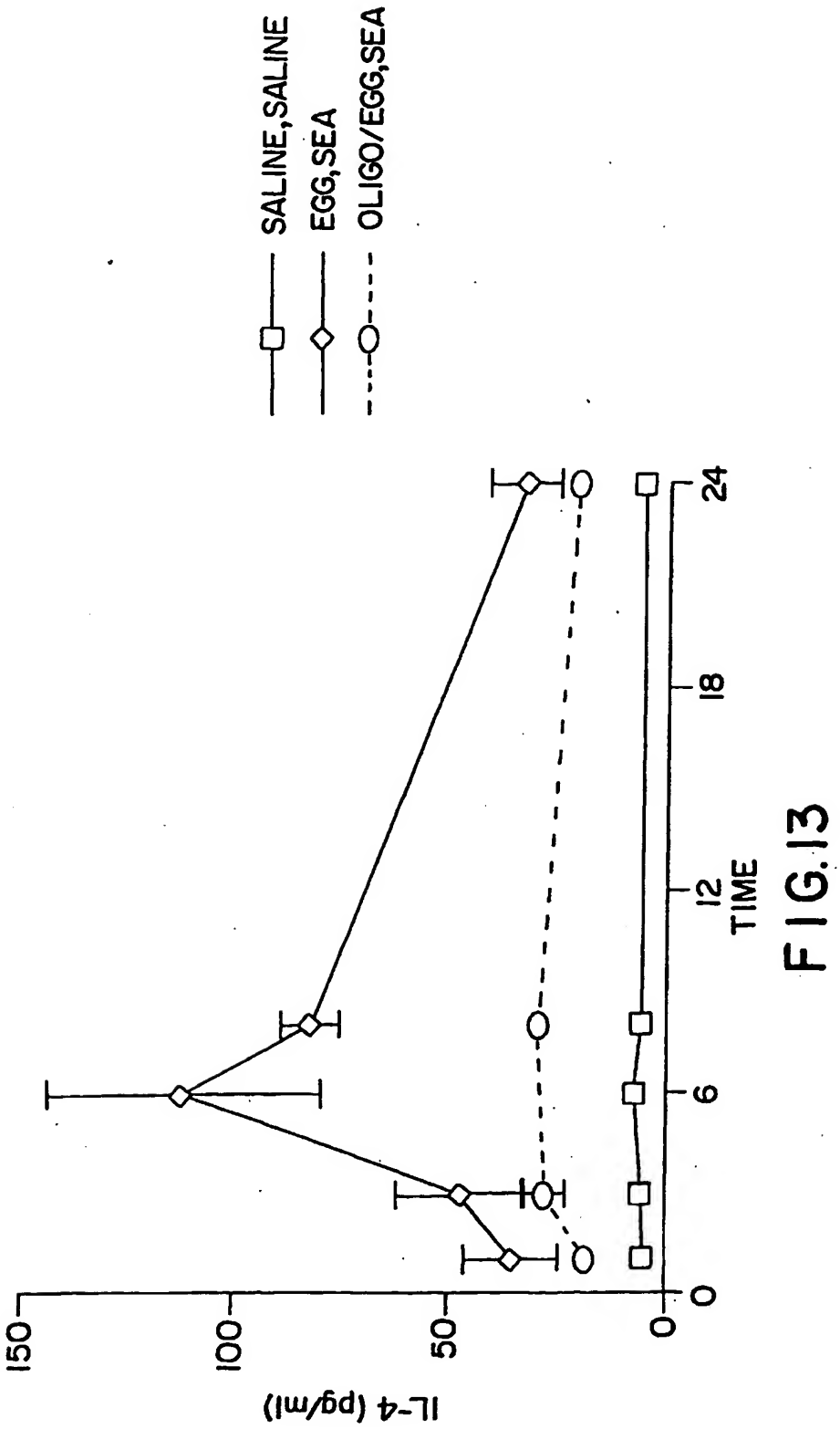
SALINE,SALINE
EGG,SEA
OLIGO/EGG,SEA
IL-4 (pg/ml)
150
100
50
0
0
6
12
18
24
TIME

FIG.13

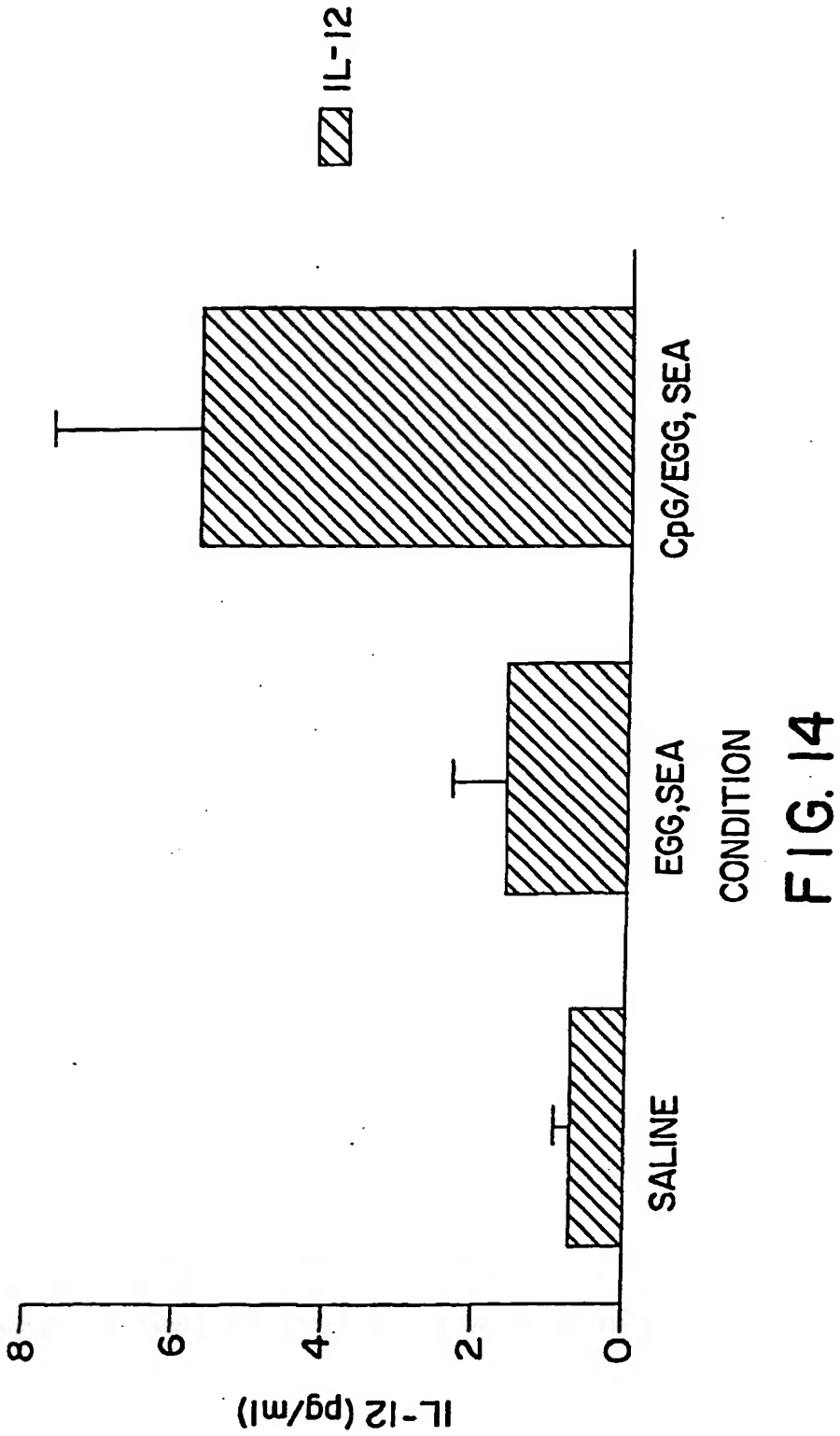

FIG. 14

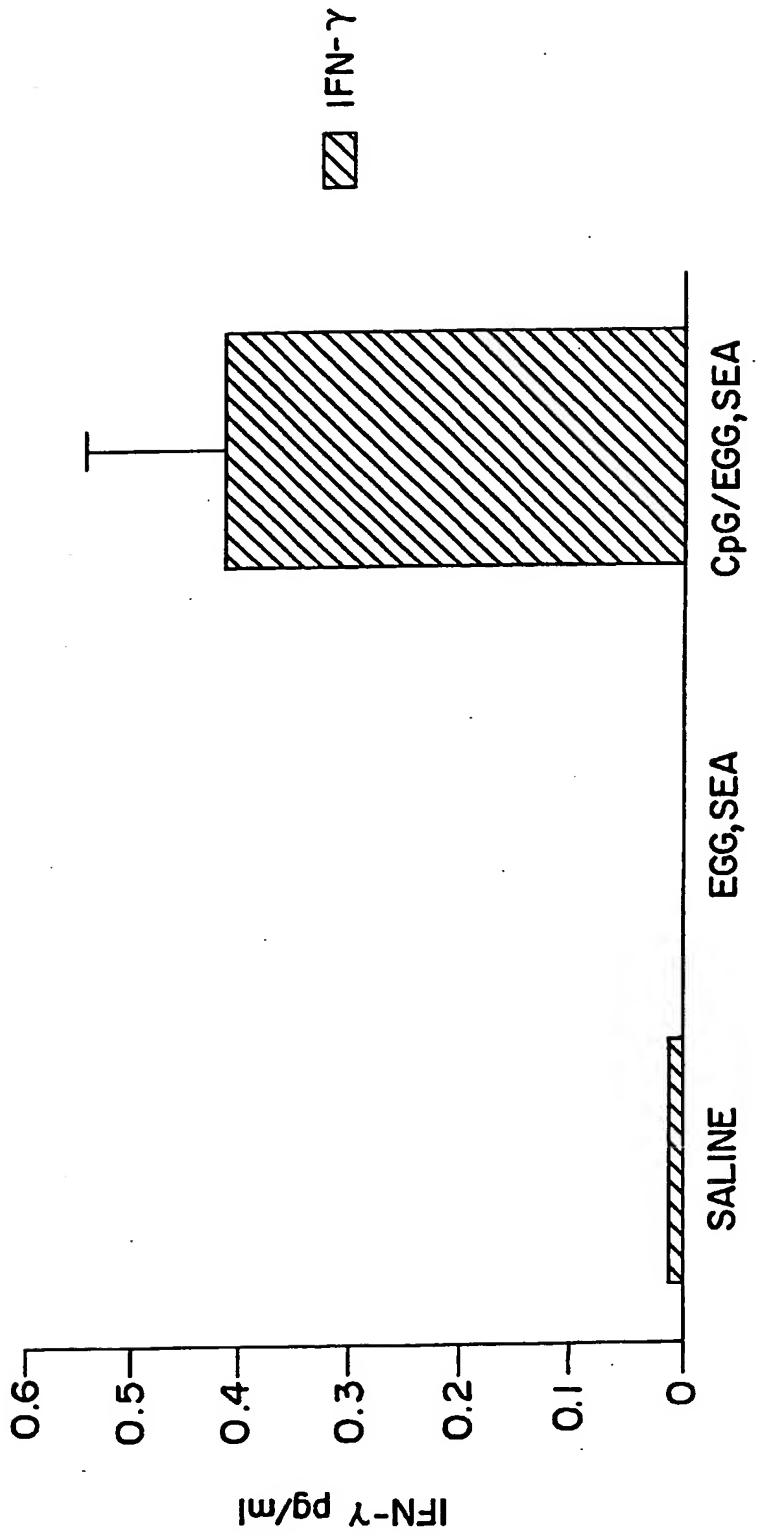
IFN-γ
IFN-γ pg/ml
0.6
0.5
0.4
0.3
0.2
0.1
0
SALINE
EGG,SEA
CpG/EGG,SEA
CONDITION

FIG. 15

IMMUNOSTIMULATORY NUCLEIC ACID MOLECULES

This application is a continuation-in-part of U.S. Ser. No. 08/738,652, filed Oct. 30, 1996.

The work resulting in this invention was supported in part by National Institute of Health Grant No. R29-AR42556-01. The U.S. Government may be entitled to certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates generally to oligonucleotides and more specifically to oligonucleotides which have a sequence including at least one unmethylated CpG dinucleotide which are immunostimulatory.

BACKGROUND OF THE INVENTION

In the 1970's, several investigators reported the binding of high molecular weight DNA to cell membranes (Lerner, R. A., et al. 1971. "Membrane-associated DNA in the cytoplasm of diploid human lymphocytes". *Proc. Natl. Acad. Sci. USA* 68:1212; Agrawal, S. K., R. W. Wagner, P. K. McAllister, and B. Rosenberg. 1975. "Cell-surface-associated nucleic acid in tumorigenic cells made visible with platinum-pyrimidine complexes by electron microscopy". *Proc. Natl. Acad. Sci. USA* 72:928). In 1985, Bennett et al. presented the first evidence that DNA binding to lymphocytes is similar to a ligand receptor interaction: binding is saturable, competitive, and leads to DNA endocytosis and degradation into oligonucleotides (Bennett, R. M., G. T. Gabor, and M. M. Merritt. 1985. "DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA". *J. Clin. Invest.* 76:2182). Like DNA, oligodeoxyribonucleotides (ODNs) are able to enter cells in a saturable, sequence independent, and temperature and energy dependent fashion (reviewed in Jaroszewski, J. W., and J. S. Cohen. 1991. "Cellular uptake of antisense oligodeoxynucleotides". *Advanced Drug Delivery Reviews* 6:235; Akhtar, S., Y. Shoji, and R. L. Juliano. 1992. "Pharmaceutical aspects of the biological stability and membrane transport characteristics of antisense oligonucleotides". In: *Gene Regulation: Biology of Antisense RNA and DNA*. R. P. Erickson, and J. G. Izant, eds. Raven Press, Ltd. New York, pp. 133; and Zhao, Q., T. Waldschmidt, E. Fisher, C. J. Herrera, and A. M. Krieg., 1994. "Stage specific oligonucleotide uptake in murine bone marrow B cell precursors". *Blood*, 84:3660). No receptor for DNA or ODN uptake has yet been cloned, and it is not yet clear whether ODN binding and cell uptake occurs through the same or a different mechanism from that of high molecular weight DNA.

Lymphocyte ODN uptake has been shown to be regulated by cell activation. Spleen cells stimulated with the B cell mitogen LPS had dramatically enhanced ODN uptake in the B cell population, while spleen cells treated with the T cell mitogen Con A showed enhanced ODN uptake by T but not B cells (Krieg, A. M., F. Gmelig-Meyling, M. F. Gourley, W. J. Kisch, L. A. Chrisey, and A. D. Steinberg. 1991. "Uptake of oligodeoxyribonucleotides by lymphoid cells is heterogeneous and inducible". *Antisense Research and Development* 1:161).

Several polynucleotides have been extensively evaluated as biological response modifiers. Perhaps the best example is poly (I,C) which is a potent inducer of IFN production as well as a macrophage activator and inducer of NK activity (Talmadge, J. E., J. Adams, H. Phillips, M. Collins, B. Lenz, M. Schneider, E. Schlick, R. Ruffmann, R. H. Wiltrout, and M. A. Chirigos. 1985. "Immunomodulatory effects in mice of polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose". *Cancer Res.* 45:1058; Wiltrout, R. H., R. R. Salup, T. A. Twilley, and J. E. Talmadge. 1985. "Immunomodulation of natural killer activity by polyribonucleotides". *J. Biol. Resp. Mod.* 4:512; Krown, S. E. 1986. "Interferons and interferon inducers in cancer treatment". *Sem. Oncol.* 13:207; and Ewel, C. H., S. J. Urba, W. C. Kopp, J. W. Smith II, R. G. Steis, J. L. Rossio, D. L. Longo, M. J. Jones, W. G. Alvord, C. M. Pinsky, J. M. Beveridge, K. L. McNitt, and S. P. Creekmore. 1992. "Polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose in combination with interleukin-2 in patients with cancer: clinical and immunological effects". *Canc. Res.* 52:3005). It appears that this murine NK activation may be due solely to induction of IFN-β secretion (Ishikawa, R., and C. A. Biron. 1993. "IFN induction and associated changes in splenic leukocyte distribution". *J. Immunol.* 150:3713). This activation was specific for the ribose sugar since deoxyribose was ineffective. Its potent in vitro antitumor activity led to several clinical trials using poly (I,C) complexed with poly-L-lysine and carboxymethylcellulose (to reduce degradation by RNAse) (Talmadge, J. E., et al., 1985. cited supra; Wiltrout, R. H., et al., 1985. cited supra); Krown, S. E., 1986. cited supra); and Ewel, C. H., et al., 1992. cited supra). Unfortunately, toxic side effects have thus far prevented poly (I,C) from becoming a useful therapeutic agent.

Guanine ribonucleotides substituted at the C8 position with either a bromine or a thiol group are B cell mitogens and may replace "B cell differentiation factors" (Feldbush, T. L., and Z. K. Ballas. 1985. "Lymphokine-like activity of 8-mercaptoguanosine: induction of T and B cell differentiation". *J. Immunol.* 134:3204; and Goodman, M. G. 1986. "Mechanism of synergy between T cell signals and C8-substituted guanine nucleosides in humoral immunity: B lymphotropic cytokines induce responsiveness to 8-mercaptoguanosine". *J. Immunol.* 136:3335). 8-mercaptoguanosine and 8-bromoguanosine also can substitute for the cytokine requirement for the generation of MHC restricted CTL (Feldbush, T. L., 1985. cited supra), augment murine NK activity (Koo, G. C., M. E. Jewell, C. L. Manyak, N. H. Sigal, and L. S. Wicker. 1988. "Activation of murine natural killer cells and macrophages by 8-bromoguanosine". *J. Immunol.* 140:3249), and synergize with IL-2 in inducing murine LAK generation (Thompson, R. A., and Z. K. Ballas. 1990. "Lymphokine-activated killer (LAK) cells. V. 8-Mercaptoguanosine as an IL-2-sparing agent in LAK generation". *J. Immunol.* 145:3524). The NK and LAK augmenting activities of these C8-substituted guanosines appear to be due to their induction of IFN (Thompson, R. A., et al. 1990. cited supra). Recently, a 5' triphosphorylated thymidine produced by a mycobacterium was found to be mitogenic for a subset of human γδ T cells (Constant, P., F. Davodeau, M.-A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J.-J. Fournie. 1994. "Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands" *Science* 264:267). This report indicated the possibility that the immune system may have evolved ways to preferentially respond to microbial nucleic acids.

Several observations suggest that certain DNA structures may also have the potential to activate lymphocytes. For example, Bell et al. reported that nucleosomal protein-DNA complexes (but not naked DNA) in spleen cell supernatants caused B cell proliferation and immunoglobulin secretion (Bell, D.A., B. Morrison, and P. VandenBygaart. 1990.

"Immunogenic DNA-related factors". *J. Clin. Invest.* 85:1487). In other cases, naked DNA has been reported to have immune effects. For example, Messina et al. have recently reported that 260 to 800 bp fragments of poly (dG).(dC) and poly (dG.dC) were mitogenic for B cells (Messina, J. P., G. S. Gilkeson, and D. S. Pisetsky. 1993. "The influence of DNA structure on the in vitro stimulation of murine lymphocytes by natural and synthetic polynucleotide antigens". *Cell. Immunol* 147:148). Tokunaga, et al. have reported that dG.dC induces γ-IFN and NK activity (Tokunaga, S. Yamamoto, and K. Namba. 1988. "A synthetic single-stranded DNA, poly(dG, dC), induces interferon-α/b and -g, augments natural killer activity, and suppresses tumor growth" Jpn. *J. Cancer Res.* 79:682). Aside from such artificial homopolymer sequences, Pisetsky et al. reported that pure mammalian DNA has no detectable immune effects, but that DNA from certain bacteria induces B cell activation and immunoglobulin secretion (Messina, J. P., G. S. Gilkeson, and D. S. Pisetsky. 1991. "Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA". *J. Immunol.* 147:1759). Assuming that these data did not result from some unusual contaminant, these studies suggested that a particular structure or other characteristic of bacterial DNA renders it capable of triggering B cell activation. Investigations of mycobacterial DNA sequences have demonstrated that ODN which contain certain palindrome sequences can activate NK cells (Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992. "Unique palindromic sequences in synthetic oligonucleotides are required to induce INF and augment INF-mediated natural killer activity". *J. Immunol.* 148:4072; Kuramoto, E., 0. Yano, Y. Kimura, M. Baba, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1992. "Oligonucleotide sequences required for natural killer cell activation". *Jpn. J Cancer Res.* 83:1128).

Several phosphorothioate modified ODN have been reported to induce in vitro or in vivo B cell stimulation (Tanaka, T., C. C. Chu, and W. E. Paul. 1992. "An antisense oligonucleotide complementary to a sequence in Ig2b increases g2b germline transcripts, stimulates B cell DNA synthesis, and inhibits immunoglobulin secretion". *J. Exp. Med.* 175:597; Branda, R. F., A. L. Moore, L. Mathews, J. J. McCormack, and G. Zon. 1993. "Immune stimulation by an antisense oligomer complementary to the rev gene of HIV-1". *Biochem. Pharmacol.* 45:2037; McIntyre, K. W., K. Lombard-Gillooly, J. R. Perez, C. Kunsch, U. M. Sarmiento, J. D. Larigan, K. T. Landreth, and R. Narayanan. 1993. "A sense phosphorothioate oligonucleotide directed to the initiation codon of transcription factor NF-κB T65 causes sequence-specific immune stimulation". *Antisense Res. Develop.* 3:309; and Pisetsky, D. S., and C. F. Reich. 1993. "Stimulation of murine lymphocyte proliferation by a phosphorothioate oligonucleotide with antisense activity for herpes simplex virus". *Life Sciences* 54:101). These reports do not suggest a common structural motif or sequence element in these ODN that might explain their effects.

The cAMP response element binding protein (CREB) and activating transcription factor (ATF) or CREB/ATF family of transcription factors is a ubiquitously expressed class of transcription factors of which 11 members have so far been cloned (reviewed in de Groot, R. P., and P. Sassone-Corsi: "Hormonal control of gene expression: Multiplicity and versatility of cyclic adenosine 3',5'-monophosphate-responsive nuclear regulators". *Mol. Endocrin.* 7:145, 1993; Lee, K. A. W., and N. Masson: "Transcriptional regulation by CREB and its relatives". *Biochim. Biophys. Acta* 1174:221, 1993.). They all belong to the basic region/leucine zipper (bZip) class of proteins. All cells appear to express one or more CREB/ATF proteins, but the members expressed and the regulation of mRNA splicing appear to be tissue-specific. Differential splicing of activation domains can determine whether a particular CREB/ATF protein will be a transcriptional inhibitor or activator. Many CREB/ATF proteins activate viral transcription, but some splicing variants which lack the activation domain are inhibitory. CREB/ATF proteins can bind DNA as homo- or hetero-dimers through the cAMP response element, the CRE, the consensus form of which is the unmethylated sequence TGACGTC (binding is abolished if the CpG is methylated) (Iguchi-Ariga, S. M. M., and W. Schaffner: "CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation". *Genes & Develop.* 3:612, 1989.).

The transcriptional activity of the CRE is increased during B cell activation (Xie, H. T. C. Chiles, and T. L. Rothstein: "Induction of CREB activity via the surface Ig receptor of B cells". *J. Immunol.* 151:880, 1993.). CREB/ATF proteins appear to regulate the expression of multiple genes through the CRE including immunologically important genes such as fos, jun B, Rb-1, IL-6, IL-1 (Tsukada, J., K. Saito, W. R. Waterman, A. C. Webb, and P. E. Auron: "Transcription factors NF-IL6 and CREB recognize a common essential site in the human prointerleukin 1 gene". *Mol. Cell. Biol.* 14:7285, 1994; Gray, G. D., O. M. Hernandez, D. Hebel, M. Root, J. M. Pow-Sang, and E. Wickstrom: "Antisense DNA inhibition of tumor growth induced by c-Ha-ras oncogene in nude mice". *Cancer Res.* 53:577, 1993), IFN- (Du, W., and T. Maniatis: "An ATF/CREB binding site protein is required for virus induction of the human interferon B gene". *Proc. Natl. Acad. Sci. USA* 89:2150, 1992), TGF-1 (Asiedu, C. K., L. Scott, R. K. Assoian, M. Ehrlich: "Binding of AP-1/CREB proteins and of MDBP to contiguous sites downstream of the human TGF-B1 gene". *Biochim. Biophys. Acta* 1219:55, 1994.), TGF-2, class II MHC (Cox, P. M., and C. R. Goding: "An ATF/CREB binding motif is required for aberrant constitutive expression of the MHC class II DRa promoter and activation by SV40 T-antigen". *Nucl. Acids Res.* 20:4881, 1992.), E-selectin, GM-CSF, CD-8, the germline Ig constant region gene, the TCR V gene, and the proliferating cell nuclear antigen (Huang, D., P. M. Shipman-Appasamy, D. J. Orten, S. H. Hinrichs, and M. B. Prystowsky: "Promoter activity of the proliferating-cell nuclear antigen gene is associated with inducible CRE-binding proteins in interleukin 2-stimulated T lymphocytes". *Mol. Cell. Biol.* 14:4233, 1994.). In addition to activation through the cAMP pathway, CREB can also mediate transcriptional responses to changes in intracellular Ca^{++} concentration (Sheng, M., G. McFadden, and M. E. Greenberg: "Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB". *Neuron* 4:571, 1990).

The role of protein-protein interactions in transcriptional activation by CREB/ATF proteins appears to be extremely important. There are several published studies reporting direct or indirect interactions between NFKB proteins and CREB/ATF proteins (Whitley, et. al., (1994) *Mol. & Cell. Biol.* 14:6464; Cogswell, et al., (1994) *J. Immun.* 153:712; Hines, et al., (1993) *Oncogene* 8:3189; and Du, et al., (1993) *Cell* 74:887. Activation of CREB through the cyclic AMP pathway requires protein kinase A (PKA), which phosphorylates $CREB^{341}$ on ser^{133} and allows it to bind to a recently cloned protein, CBP (Kwok, R. P. S., J. R. Lundblad, J. C. Chrivia, J. P. Richards, H. P. Bachinger, R. G. Brennan, S. G. E. Roberts, M. R. Green, and R. H. Goodman: "Nuclear

protein CBP is a coactivator for the transcription factor CREB". *Nature* 370:223, 1994; Arias, J., A. S. Alberts, P. Brindle, F. X. Claret, T. Smea, M. Karin, J. Feramisco, and M. Montminy: "Activation of cAMP and mitogen responsive genes relies on a common nuclear factor". *Nature* 370:226, 1994.). CBP in turn interacts with the basal transcription factor TFIIB causing increased transcription. CREB also has been reported to interact with dTAFII 110, a TATA binding protein-associated factor whose binding may regulate transcription (Ferreri, K., G. Gill, and M. Montminy: "The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex". *Proc. Natl. Acad. Sci. USA* 91:1210, 1994.). In addition to these interactions, CREB/ATF proteins can specifically bind multiple other nuclear factors (Hoeffler, J. P., J. W. Lustbader, and C.-Y. Chen: "Identification of multiple nuclear factors that interact with cyclic adenosine 3',5'-monophosphate response element-binding protein and activating transcription factor-2 by protein-protein interactions". *Mol. Endocrinol.* 5:256, 1991) but the biologic significance of most of these interactions is unknown. CREB is normally thought to bind DNA either as a homodimer or as a heterodimer with several other proteins. Surprisingly, CREB monomers constitutively activate transcription (Krajewski, W., and K. A. W. Lee: "A monomeric derivative of the cellular transcription factor CREB functions; as a constitutive activator". *Mol. Cell. Biol.* 14:7204, 1994.).

Aside from their critical role in regulating cellular transcription, it has recently been shown that CREB/ATF proteins are subverted by some infectious viruses and retroviruses, which require them for viral replication. For example, the cytomegalovirus immediate early promoter, one of the strongest known mammalian promoters, contains eleven copies of the CRE which are essential for promoter function (Chang, Y.-N., S. Crawford, J. Stall, D. R. Rawlins, K.-T. Jeang, and G. S. Hayward: "The palindromic series I repeats in the simian cytomegalovirus major immediate-early promoter behave as both strong basal enhancers and cyclic AMP response elements". *J. Virol.* 64:264, 1990). At least some of the transcriptional activating effects of the adenovirus E1A protein, which induces many promoters, are due to its binding to the DNA binding domain of the CREB/ATF protein, ATF-2, which mediates E1A inducible transcription activation (Liu, F., and M. R. Green: "Promoter targeting by adenovirus E1A through interaction with different cellular DNA-binding domains". *Nature* 368:520, 1994). It has also been suggested that E1A binds to the CREB-binding protein, CBP (Arany, Z., W. R. Sellers, D. M. Livingston, and R. Eckner: "E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators". *Cell* 77:799, 1994). Human T lymphotropic virus-I (HTLV-1), the retrovirus which causes human T cell leukemia and tropical spastic paresis, also requires CREB/ATF proteins for replication. In this case, the retrovirus produces a protein, Tax, which binds to CREB/ATF proteins and redirects them from their normal cellular binding sites to different DNA sequences (flanked by G- and C-rich sequences) present within the HTLV transcriptional enhancer (Paca-Uccaralertkun, S., L.-J. Zhao, N. Adya, J. V. Cross, B. R. Cullen, I. M. Boros, and C.-Z. Giam: "In vitro selection of DNA elements highly responsive to the human T-cell lymphotropic virus type I transcriptional activator, Tax". *Mol. Cell. Biol.* 14:456, 1994; Adya, N., L.-J. Zhao, W. Huang, I. Boros, and C.-Z. Giam: "Expansion of CREB's DNA recognition specificity by Tax results from interaction with Ala-Ala-Arg at positions 282–284 near the conserved DNA-binding domain of CREB". *Proc. Natl. Acad. Sci. USA* 91:5642, 1994).

SUMMARY OF THE INVENTION

The present invention is based on the finding that certain nucleic acids containing unmethylated cytosine-guanine (CpG) dinucleotides activate lymphocytes in a subject and redirect a subject's immune response from a Th2 to a Th1 (e.g. by inducing monocytic cells and other cells to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). Based on this finding, the invention features, in one aspect, novel immunostimulatory nucleic acid compositions.

In one embodiment, the invention provides an isolated immunostimulatory nucleic acid sequence containing a CpG motif represented by the formula:

$$5'N_1X_1CGX_2N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine or thymine; N is any nucleotide and N_1+N_2 is from about 0–26 bases with the proviso that N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8–30 bases in length.

In another embodiment, the invention provides an isolated immunostimulatory nucleic acid sequence contains a CpG motif represented by the formula:

$$5'N_1X_1X_2CGX_3X_4N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3 X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and N_1+N_2 is from about 0–26 bases with the proviso that that N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8–30 bases in length.

In another embodiment, the invention provides a method of stimulating immune activation by administering the nucleic acid sequences of the invention to a subject, preferably a human. In a preferred embodiment, the immune activation effects predominantly a Th1 pattern of immune activation.

In another embodiment, the nucleic acid sequences of the invention stimulate cytokine production. In particular, cytokines such as IL-6, IL-12, IFN-γ, TNF-α and GM-CSF are produced via stimulation of the immune system using the nucleic acid sequences described herein. In another aspect, the nucleic acid sequences of the invention stimulate the lytic activity of natural killer cells (NK) and the proliferation of B cells.

In another embodiment, the nucleic acid sequences of the invention are useful as an artificial adjuvant for use during antibody generation in a mammal such as a mouse or a human.

In another embodiment, autoimmune disorders are treated by inhibiting a subject's response to CpG mediated leukocyte activation. The invention provides administration of inhibitors of endosomal acidification such as bafilomycin a, chloroquine, and monensin to ameliorate autoimmune disorders. In particular, systemic lupus erythematosus is treated in this manner.

The nucleic acid sequences of the invention can also be used to treat, prevent or ameliorate other disorders (e.g., a tumor or cancer or a viral, fungal, bacterial or parasitic infection). In addition, the nucleic acid sequences can be administered to stimulate a subject's response to a vaccine.

Furthermore, by redirecting a subject's immune response from Th2 to Th1, the claimed nucleic acid sequences can be used to treat or prevent an asthmatic disorder. In addition, the claimed nucleic acid molecules can be administered to a subject in conjunction with a particular allergen as a type of desensitization therapy to treat or prevent the occurrence of an allergic reaction associated with an asthmatic disorder.

Further, the ability of the nucleic acid sequences of the invention described herein to induce leukemic cells to enter the cell cycle supports their use in treating leukemia by increasing the sensitivity of chronic leukemia cells followed by conventional ablative chemotherapy, or by combining the nucleic acid sequences with other immunotherapies.

Other features and advantages of the invention will become more apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A–C are graphs plotting dose-dependent IL-6 production in response to various DNA sequences in T cell depleted spleen cell cultures.

FIG. 1 A. *E. coli* DNA (●) and calf thymus DNA (■) sequences and LPS (at 10x the concentration of *E. coli* and calf thymus DNA) (♦).

FIG. 1 B. Control phosphodiester oligodeoxynucleotide (ODN) $^{5'}$ATGGAAGGTCCAGTGTTCTC$^{3'}$ (SEQ ID No: 1) (■) and two phosphodiester CpG ODN $^{5'}$ATCGACCTACGTGCGTTCTC$^{3'}$ (SEQ ID No: 2) (♦) and $^{5'}$TCCATAACGTTCCTGATGCT$^{3'}$ (SEQ ID No: 3) (●).

FIG. 1 C. Control phosphorothioate ODN $^{5'}$GCTAGATGTTAGCGT$^{3'}$ (SEQ ID No: 4) (■) and two phosphorothioate CpG ODN $^{5'}$GAGAACGTCGACCTTCGAT$^{3'}$ (SEQ ID No: 5) (♦) and $^{5'}$GCATGACGTTGAGCT$^{3'}$ (SEQ ID No: 6) (●). Data present the mean±standard deviation of triplicates.

FIG. 2 is a graph plotting IL-6 production induced by CpG DNA in vivo as determined 1–8 hrs after injection. Data represent the mean from duplicate analyses of sera from two mice. BALB/c mice (two mice/group) were injected iv. with 100 μl of PBS (□) or 200 μg of CpG phosphorothioate ODN $^{5'}$TCCATGACGTTCCTGATGCT$^{3'}$ (SEQ ID No: 7) (■) or non-CpG phosphorothioate ODN $^{5'}$TCCATGAGCTTCCTGAGTCT$^{3'}$ (SEQ ID No: 8) (♦).

FIG. 3 is an autoradiograph showing IL-6 mRNA expression as determined by reverse transcription polymerase chain reaction in liver, spleen, and thymus at various time periods after in vivo stimulation of BALB/c mice (two mice/group) injected iv with 100 μl of PBS, 200 μg of CpG phosphorothioate ODN $^{5'}$TCCATGACGTTCCTGATGCT$^{3'}$ (SEQ ID No: 7) or non-CpG phosphorothioate ODN $^{5'}$TCCATGAGCTTCCTGAGTCT$^{3'}$ (SEQ ID No: 8).

FIG. 4A is a graph plotting dose-dependent inhibition of CpG-induced IgM production by anti-IL-6. Splenic B-cells from DBA/2 mice were stimulated with CpG ODN $^{5'}$TCCAAGACGTTCCTGATGCT$^{3'}$ (SEQ ID No: 9) in the presence of the indicated concentrations of neutralizing anti-IL-6 (♦) or isotype control Ab (●) and IgM levels in culture supernatants determined by ELISA. In the absence of CpG ODN, the anti-IL-6 Ab had no effect on IgM secretion (■).

FIG. 4B is a graph plotting the stimulation index of CpG-induced splenic B cells cultured with anti-IL-6 and CpG S-ODN $^{5'}$TCCATGACGTTCCTGATGCT$^{3'}$ (SEQ ID No: 7) (♦) or anti-IL-6 antibody only (■). Data present the mean±standard deviation of triplicates.

FIG. 5 is a bar graph plotting chloramphenicol acetyltransferase (CAT) activity in WEHI-231 cells transfected with a promoter-less CAT construct (pCAT), positive control plasmid (RSV), or IL-6 promoter-CAT construct alone or cultured with CpG $^{5'}$CCATGACGTTCCTGATGCT$^{3'}$ (SEQ ID No: 7) or non-CpG $^{5'}$TCCATGAGCTTCCTGAGTCT$^{3'}$ (SEQ ID No: 8) phosphorothioate ODN at the indicated concentrations. Data present the mean of triplicates.

FIG. 6 is a schematic overview of the immune effects of the immunostimulatory unmethylated CpG containing nucleic acids, which can directly activate both B cells and monocytic cells (including macrophages and dendritic cells) as shown. The immunostimulatory oligonucleotides do not directly activate purified NK cells, but render them competent to respond to IL-12 with a marked increase in their IFN-γ production. By inducing IL-12 production and the subsequent increased IFN-γ secretion by NK cells, the immunostimulatory nucleic acids promote a Th1 type immune response. No direct activation of proliferation of cytokine secretion by highly purified T cells has been found. However, the induction of Th1 cytokine secretion by the immunostimulatory oligonucleotides promotes the development of a cytotoxic lymphocyte response.

FIG. 7 is an autoradiograph showing NFkB mRNA induction in monocytes treated with *E. coli* (EC) DNA (containing unmethylated CpG motifs), control (CT) DNA (containing no unmethylated CpG motifs) and lipopolysaccharide (LPS) at various measured times, 15 and 30 minutes after contact.

FIG. 8A shows the results from a flow cytometry study using mouse B cells with the dihydrorhodamine 123 dye to determine levels of reactive oxygen species. The dye only sample in Panel A of the figure shows the background level of cells positive for the dye at 28.6%. This level of reactive oxygen species was greatly increased to 80% in the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the CpG oligo (TCCATGACGTTCCTGACGTT SEQ ID No. 10) also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the CpGs were switched (TCCATGAGCTTCCTGAGTGCT SEQ ID NO. 11) did not show this significant increase in the level of reactive oxygen species (Panel E).

FIG. 8B shows the results from a flow cytometry study using mouse B cells in the presence of chloroquine with the dihydrorhodamine 123 dye to determine levels of reactive oxygen species. Chloroquine slightly lowers the background level of reactive oxygen species in the cells such that the untreated cells in Panel A have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with CpG DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and ionomycin (Panel E).

FIG. 9 is a graph plotting lung lavage cell count over time. The graph shows that when the mice are initially injected with *Schistosoma mansoni* eggs "egg", which induces a Th2 immune response, and subsequently inhale *Schistosoma mansoni* egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given CpG oligo (SEQ ID NO. 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation of SEA (open triangles).

FIG. 10 is a graph plotting lung lavage eosinophil count over time. Again, the graph shows that when the mice are initially injected with egg and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given CpG oligo (SEQ

ID NO. 10) along with egg, the inflammatory cells in the lung are not increased by subsequent inhalation of the SEA (open triangles).

FIG. 11 is a bar graph plotting the effect on the percentage of macrophage, lymphocyte, neutrophil and eosinophil cells induced by exposure to saline alone; egg, then SEA; egg and SEQ ID No. 11, then SEA; and egg and control oligo (SEQ ID No. 11), then SEA. When the mice are treated with the control oligo at the time of the initial exposure to the egg, there is little effect on the subsequent influx of eosinophils into the lungs after inhalation of SEA. Thus, when mice inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a CpG oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes the increase in eosinophils when the mice inhale the egg antigen on day 14.

FIG. 12 is a bar graph plotting eosinophil count in response to injection of various amounts of the protective oligo SEQ ID No. 10.

FIG. 13 is a graph plotting interleukin 4 (IL-4) production (pg/ml) in mice over time in response to injection of egg, then SEA (open diamond); egg and SEQ ID No. 10, then SEA (open circle); or saline, then saline (open square). The graph shows that the resultant inflammatory response correlates with the levels of the Th2 cytokine IL-4 in the lung.

FIG. 14 is a bar graph plotting interleukin 12 (IL-12) production (pg/ml) in mice over time in response to injection of saline; egg, then SEA; or SEQ ID No. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated CpG motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune response.

FIG. 15 is a bar graph plotting interferon gamma (IFN-γ) production (pg/ml) in mice over time in response to injection of saline; egg, then saline; or SEQ ID No. 10 and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated CpG motif can also redirect the cytokine response of the lung to production of IFN-g, indicating a Th1 type of immune response.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the following terms and phrases shall have the meanings set forth below:

An "allergen" refers to a substance that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g. penicillin). Examples of natural, animal and plant allergens include proteins specific to the following genuses: Canine (*Canis familiaris*); Dermatophagoides (e.g. *Dermatophagoides farinae*); Felis (*Felis domesticus*); Ambrosia (*Ambrosia artemiisfolia;* Lolium (e.g. *Lolium perenne* or *Lolium multiflorum*); Cryptomeria (*Cryptomeria japonica*); Alternaria (*Alternaria alternata*); Alder; Alnus (*Alnus gultinosa*); Betula (*Betula verrucosa*); Quercus (*Quercus alba*); Olea (*Olea europa*); Artemisia (*Artemisia vulgaris*); Plantago (e.g. *Plantago lanceolata*); Parietaria (e.g. *Parietaria officinalis* or *Parietaria judaica*); Blattella (e.g. *Blattella germanica*); Apis (e.g. *Apis multiflorum*); Cupressus (e.g. *Cupressus sempervirens, Cupressus arizonica* and *Cupressus macrocarpa*); Juniperus (e.g. *Juniperus sabinoides, Juniperus virginiana, Juniperus communis* and *Juniperus ashei*); Thuya (e.g. *Thuya orientalis*); Chamaecyparis (e.g. *Chamaecyparis obtusa*); Periplaneta (e.g. *Periplaneta americana*); Agropyron (e.g. *Agropyron repens*); Secale (e.g. *Secale cereale*); Triticum (e.g. *Triticum aestivum*); Dactylis (e.g. *Dactylis glomerata*); Festuca (e.g. *Festuca elatior*); Poa (e.g. *Poa pratensis* or *Poa compressa*); Avena (e.g. *Avena sativa*); Holcus (e.g. *Holcus lanatus*); Anthoxanthum (e.g. *Anthoxanthum odoratum*); Arrhenatherum (e.g. *Arrhenatherum elatius*); Agrostis (e.g. *Agrostis alba*); Phleum (e.g. *Phleum pratense*); Phalaris (e.g. *Phalaris arundinacea*); Paspalum (e.g. *Paspalum notatum*); Sorghum (e.g. *Sorghum halepensis*); and Bromus (e.g. *Bromus inermis*).

An "allergy" refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include eczema, allergic rhinitis or coryza, hay fever, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions.

"Asthma"—refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

An "immune system deficiency" shall mean a disease or disorder in which the subject's immune system is not functioning in normal capacity or in which it would be useful to boost a subject's immune response for example to eliminate a tumor or cancer (e.g. tumors of the brain, lung (e.g. small cell and non-small cell), ovary, breast, prostate, colon, as well as other carcinomas and sarcomas) or an infection in a subject.

Examples of infectious virus include: Retroviridae (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g., polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g., strains that cause gastroenteritis); Togaviridae (e.g., equine encephalitis viruses, rubella viruses); Flaviridae (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g., coronaviruses); Rhabdoviridae (e.g., vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g., ebola viruses); Paramyxoviridae (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g., influenza viruses); Bungaviridae (e.g., Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g., reoviruses, orbiviuises and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvovirusies); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses'); Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g., African swine fever virus); and unclassified viruses (e.g., the etiological agents of Spongiform encephalopathies, the agent of delta hepatities (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

Examples of infectious bacteria include: *Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia,* Mycobacteria sps (e.g. *M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae*), *Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A

Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), Streptococcus (viridans group), *Streptococcus faecalis, Streptococcus bovis,* Streptococcus (anaerobic sps.), *Streptococcus pneumoniae,* pathogenic Campylobacter sp., Enterococcus sp., *Haemophilus influenzae, Bacillus antracis, Corynebacterium diphtheriae,* corynebacterium sp., *Erysipelothrix rhusiopathiae, Clostridium perfringers, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasturella multocida,* Bacteroides sp., *Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue,* Leptospira, and *Actinomyces israelli.*

Examples of infectious fungi include: *Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans.* Other infectious organisms (i.e., protists) include: *Plasmodium falciparum* and *Toxoplasma gondii.*

An "immunostimulatory nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An immunostimulatory nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have increased immune activity.

In one preferred embodiment the invention provides an isolated immunostimulalory nucleic acid sequence containing a CpG motif represented by the formula:

$$5'N_1X_1CGX_2N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine or thymine; N is any nucleotide and N_1+N_2 is from about 0–26 bases with the proviso that N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8–30 bases in length.

In another embodiment the invention provides an isolated immunostimulatory nucleic acid sequence contains a CpG motif represented by the formula:

$$5'N_1X_1X_2CGX_3X_4N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3 X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and N_1+N_2 is from about 0–26 bases with the proviso that that N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8–30 bases in length.

Preferably the immunostimulatory nucleic acid sequences of the invnetion include X_1X_2 selected from the group consisting of GpT, GpG, GpA and ApA and X_3X_4 is selected from the group consisting of TpT, CpT and GpT (see for example, Table 5). For facilitating uptake into cells, CpG containing immunostimulatory nucleic acid molecules are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size (even many kb long) are immunostimulatory if sufficient immunostimulatory motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include a a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic CpG motif is not a palindrome. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone modification. For example, the modification is a phosphorothioate or phosphorodithioate modification. More particularly, the phosphate backbone modification occurs at the 5' end of the nucleic acid for example, at the first two nucleotides of the 5' end of the nucleic acid. Further, the phosphate backbone modification may occur at the 3' end of the nucleic acid for example, at the last five nucleotides of the 3' end of the nucleic acid.

Preferably the immunostimulatory CpG DNA is in the range of between 8 to 30 bases in size when it is an oligonucleotide. Alternatively, CpG dinucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides. Preferred immunostimulatory nucleic acid molecules (e.g. for use in increasing the effectiveness of a vaccine or to treat an immune system deficiency by stimulating an antibody (i.e., humoral) response in a subject) have a relatively high stimulation index with regard to B cell, monocyte and/or natural killer cell responses (e.g. cytokine, proliferative, lytic or other responses).

The nucleic acid sequences of the invention stimulate cytokine production in a subject for example. Cytokines include but are not limited to IL-6, IL-12, IFN-γ, TNF-α and GM-CSF. Exemplary sequences include: TCCATGTCGCTCCTGATGCT (SEQ ID NO: 37), TCCATGTCGTTCCTGATGCT (SEQ ID NO: 38), and TCGTCGTTTTGTCGTTTTGTCGT (SEQ ID NO:46).

The nucleic acid sequences of the invention are also useful for stimulating natural killer cell (NK) lytic acitivity in a subject such as a human. Specific, but non-limiting examples of such sequences include: TCGTCGTTGTCGTTGTCGTT (SEQ ID NO: 47), TCGTCGTTTTGTCGTTTGTCGTT (SEQ ID NO:46), TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:49), GCGTGCGTTGTCGTTGTCGTT (SEQ ID NO:56), TGTCGTTTGTCGTTTGTCGTT (SEQ ID NO:48), TGTCGTTGTCGTTGTCGTT (SEQ ID NO:50) and TCGTCGTCGTCGTT (SEQ ID NO:51).

The nucleic acid sequences of the invention are also useful for stimulating B cell proliferation in a subject such as a human. Specific, but non-limiting examples of such sequences include: TCCTGTCGTTCCTTGTCGTT(SEQ ID NO:52), TCCTGTCGTTTTTTGTCGTT (SEQ ID NO:53), TCGTCGCTGTCTGCCCTTCTT(SEQ ID NO:54),TCGTCGCTGTTGTCGTTTCTT (SEQ ID NO:64),TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46),TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:49) and TGTCGTTGTCGTTGTCGTT (SEQ ID NO:50).

In another aspect, the nucleic acid sequences of the invention are useful as an adjuvant for use during antibody production in a mammal. Specific, but non-limiting examples of such sequences include: TCCATGACGTTCCTGACGTT (SEQ ID NO.10), GTCG(T/C)T and TGTCG(T/C)T. Furthermore, the claimed nucleic acid sequences can be administered to treat or prevent the symptoms of an asthmatic disorder by redirecting a subject's immune response from Th2 to Th1. An exemplary sequence includes TCCATGACGTTCCTGACGTT (SEQ ID NO.10).

The stimulation index of a particular immunostimulatory CpG DNA can be tested in various immune cell assays. Preferably, the stimulation index of the immunostimulatory CpG DNA with regard to B-cell proliferation is at least about

5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of ^{3}H uridine in a murine B cell culture, which has been contacted with a 20 μM of ODN for 20 h at 37° C. and has been pulsed with 1 μCi of ^{3}H uridine; and harvested and counted 4h later as described in detail in Example 1. For use in vivo, for example to treat an immune system deficiency by stimulating a cell-mediated (local) immune response in a subject, it is important that the immunostimulatory CpG DNA be capable of effectively inducing cytokine secretion by monocytic cells and/or Natural Killer (NK) cell lytic activity.

Preferred immunostimulatory CpG nucleic acids should effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml of IL-6, 200 pg/ml IL-12, depending on the therapeutic indication, as determined by the assays described in Example 12. Other preferred immunostimulatory CpG DNAs should effect at least about 10%, more preferably at least about 15% and most preferably at least about 20% YAC-1 cell specific lysis or at least about 30, more preferably at least about 35 and most preferably at least about 40% 2C11 cell specific lysis as determined by the assay described in detail in Example 4.

A "nucleic acid" or "DNA" means multiple nucleotides (i.e., molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). As used herein, the term refers to ribonucleotides as well as oligodeoxyribonucleotides. The term shall also include polynucleosides (i.e., a polynucleotide minus the phosphate) and any other organic base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis).

A "nucleic acid delivery complex" shall mean a nucleic acid molecule associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g. B-cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells). Examples of nucleic acid delivery complexes include nucleic acids associated with: a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes must be sufficiently stable in vivo to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable under appropriate conditions within the cell so that the nucleic acid is released in a functional form.

"Palindromic sequence" shall mean an inverted repeat (i.e., a sequence such as ABCDEED'CB'A' in which A and A' are bases capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double stranded structures.

A "stabilized nucleic acid molecule" shall mean a nucleic acid molecule that is relatively resistant to in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG containing nucleic acid molecules that are tens to hundreds of kbs long are relatively resistant to in vivo degradation. For shorter immunostimulatory nucleic acid molecules, secondary structure can stabilize and increase their effect. For example, if the 3' end of a nucleic acid molecule has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop structure, then the nucleic acid molecule becomes stabilized and therefore exhibits more activity.

Preferred stabilized nucleic acid molecules of the instant invention have a modified backbone. For use in immune stimulation, especially preferred stabilized nucleic acid molecules are phosphorothioate (i.e., at least one of the phosphate oxygens of the nucleic acid molecule is replaced by sulfur) or phosphorodithioate modified nucleic acid molecules. More particularly, the phosphate backbone modification occurs at the 5' end of the nucleic acid for example, at the first two nucleotides of the 5' end of the nucleic acid. Further, the phosphate backbone modification may occur at the 3' end of the nucleic acid for example, at the last five nucleotides of the 3' end of the nucleic acid. In addition to stabilizing nucleic acid molecules, as reported further herein, phosphorothioate-modified nucleic acid molecules (including phosphorodithioate-modified) can increase the extent of immune stimulation of the nucleic acid molecule, which contains an unmethylated CpG dinucleotide as shown herein. International Patent Application Publication Number: WO 95/26204 entitled "Immune Stimulation By Phosphorothioate Oligonucleotide Analogs" also reports on the non-sequence specific immunostimulatory effect of phosphorothioate modified oligonucleotides. As reported herein, unmethylated CpG containing nucleic acid molecules having a phosphorothioate backbone have been found to preferentially activate B-cell activity, while unmethylated CpG containing nucleic acid molecules having a phosphodiester backbone have been found to preferentially activate monocytic (macrophages, dendritic cells and monocytes) and NK cells. Phosphorothioate CpG oligonucleotides with preferred human motifs, are also strong activators of monocytic and NK cells.

Other stabilized nucleic acid molecules include: nonionic DNA analogs, such as alkyl- and aryl-phosphonates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Nucleic acid molecules which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

A "subject" shall mean a human or vertebrate animal including a dog, cat, horse, cow, pig, sheep, goat, chicken, monkey, rat, and mouse.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Preferred vectors are those capable of autonomous replication and expression of nucleic acids to which they are linked (e.g., an episome). Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

Certain Unmethylated CpG Containing Nucleic Acids Have B Cell Stimulatory Activity As Shown in vitro and in vivo

In the course of investigating the lymphocyte stimulatory effects of two antisense oligonucleotides specific for endog-

enous retroviral sequences, using protocols described in the attached Examples 1 and 2, it was surprisingly found that two out of twenty-four "controls" (including various scrambled, sense, and mismatch controls for a panel of "antisense" ODN) also mediated B cell activation and IgM secretion, while the other "controls" had no effect.

Two observations suggested that the mechanism of this B cell activation by the "control" ODN may not involve antisense effects 1) comparison of vertebrate DNA sequences listed in GenBank showed no greater homology than that seen with non-stimulatory ODN and 2) the two controls showed no hybridization to Northern blots with 10 μg of spleen poly A+ RNA. Resynthesis of these ODN on a different synthesizer or extensive purification by polyacrylamide gel electrophoresis or high pressure liquid chromatography gave identical stimulation, eliminating the possibility of an impurity. Similar stimulation was seen using B cells from C3H/HeJ mice, eliminating the possibility that lipopolysaccharide (LPS) contamination could account for the results.

The fact that two "control" ODN caused B cell activation similar to that of the two "antisense" ODN raised the possibility that all four ODN were stimulating B cells through some non-antisense mechanism involving a sequence motif that was absent in all of the other nonstimulatory control ODN. In comparing these sequences, it was discovered that all of the four stimulatory ODN contained CpG dinucleotides that were in a different sequence context from the nonstimulatory control.

To determine whether the CpG motif present in the stimulatory ODN was responsible for the observed stimulation, over 300 ODN ranging in length from 5 to 42 bases that contained methylated, unmethylated, or no CpG dinucleotides in various sequence contexts were synthesized. These ODNs, including the two original "controls" (ODN 1 and 2) and two originally synthesized as "antisense" (ODN 3D and 3M; Krieg, A. M. *J. Immunol.* 143:2448 (1989)), were then examined for in vitro effects on spleen cells (representative sequences are listed in Table 1). Several ODN that contained CpG dinucleotides induced B cell activation and IgM secretion; the magnitude of this stimulation typically could be increased by adding more CpG dinucleotides (Table 1; compare ODN 2 to 2a or 3D to 3Da and 3Db). Stimulation did not appear to result from an antisense mechanism or impurity. ODN caused no detectable proliferation of γδ or other T cell populations.

Mitogenic ODN sequences uniformly became nonstimulatory if the CpG dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the CpG dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b,2b,3Dd, and 3Mb). Partial methylation of CpG motifs caused a partial loss of stimulatory effect (compare 2a to 2c, Table 1). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a CpG motif is the essential element present in ODN that activate B cells.

In the course of these studies, it became clear that the bases flanking the CpG dinucleotide played an important role in determining the murine B cell activation induced by an ODN. The optimal stimulatory motif was determined to consist of a CpG flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the CpG motif closer to this ideal improved stimulation (e.g. Table 1, compare ODN 2 to 2e; 3M to 3Md) while mutations that disturbed the motif reduced stimulation (e.g. Table 1, compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the CpG motif did not reduce stimulation (e.g. Table 1, compare ODN 1 to id; 3D to 3Dg; 3M to 3Me). For activation of human cells, the best flanking bases are slightly different (See Table 5).

Of those tested, ODNs shorter than 8 bases were nonstimulatory (e.g. Table 1, ODN 4e). Among the forty-eight 8 base ODN tested, a highly stimulatory sequence was identified as TCAACGTT (ODN 4) which contains the self complementary "palindrome" AACGTT. In further optimizing this motif, it was found that ODN containing Gs at both ends showed increased stimulation, particularly if the ODN were rendered nuclease resistant by phosphorothioate modification of the terminal internucleotide linkages. ODN 1585 (5' GGGGTCAACGTTCAGGGGGG 3' (SEQ ID NO: 12)), in which the first two and last five internucleotide linkages are phosphorothioate modified caused an average 25.4 fold increase in mouse spleen cell proliferation compared to an average 3.2 fold increase in proliferation induced by ODN 1638, which has the same sequence as ODN 1585 except that the 10 Gs at the two ends, are replaced by 10 As. The effect of the G-rich ends is cis; addition of an ODN with poly G ends, but no CpG motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated CpG were found to be more immunostimulatory.

Other octamer ODN containing a 6 base palindrome with a TpC dinucleotide at the 5' end were also active (e.g. Table 1, ODN 4b,4c). Other dinucleotides at the 5' end gave reduced stimulation (e.g., ODN 4f; all sixteen possible dinucleotides were tested). The presence of a 3' dinucleotide was insufficient to compensate for the lack of a 5' dinucleotide (e.g., Table 1, ODN 4g). Disruption of the palindrome eliminated stimulation in octamer ODN (e.g., Table 1, ODN 4h), but palindromes were not required in longer ODN.

TABLE 1

Olionucleotide Stimulation of Mouse B Cells

ODN		Sequence (5'to 3')†	Stimulation Index' ³H Uridine	IgM Production
1	(SEQ ID NO:91)	GCTAGACGTTAGCGT	6.1 ± 0.8	17.9 ± 3.6
1a	(SEQ. ID NO:4)	......T......_.	1.2 ± 0.2	1.7 ± 0.5
1b	(SEQ ID NO:13)	......Z....._._	1.2 ± 0.1	1.8 ± 0.0
1c	(SEQ ID NO:14)	....._._....Z..	10.3 ± 4.4	9.5 ± 1.8

TABLE 1-continued

Olionucleotide Stimulation of Mouse B Cells

ODN	Sequence (5'to 3')†	Stimulation Index' ^{3}H Uridine	Stimulation Index' IgM Production
1d (SEQ ID NO:92)	..AT..__..GAGC.	13.0 ± 2.3	18.3 ± 7.5
2 (SEQ ID NO:1)	ATGGAAGGTCCAGCGTTCTC	2.9 ± 0.2	13.6 ± 2.0
2a (SEQ ID NO:15)	..C_.CTC._G.._____	7.7 ± 0.8	24.2 ± 3.2
2b (SEQ ID NO:16)	..E..CTC.EG..E.....	1.6 ± 0.5	2.8 ± 2.2
2c (SEQ ID NO:17)	..E..CTC._G.._____	3.1 ± 0.6	7.3 ± 1.4
2d (SEQ ID NO:18)	..C_.CTC._G.._____..E..	7.4 ± 1.4	27.7 ± 5.4
2e (SEQ ID NO:19)	..........A_____	5.6 ± 2.0	ND
3D (SEQ ID NO:20)	GAGAACGCTGGACCTTCCAT	4.9 ± 0.5	19.9 ± 3.6
3Da (SEQ ID NO:21)	....._____._C.....	6.6 ± 1.5	33.9 ± 6.8
3Db (SEQ ID NO:22)	....._____.C_....._G..	10.1 ± 2.8	25.4 ± 0.8
3Dc (SEQ ID NO:23)	...C.A.....	1.0 ± 0.1	1.2 ± 0.5
3Dd (SEQ ID NO:24)	.....E.....	1.2 ± 0.2	1.0 ± 0.4
3De (SEQ ID NO:25)	....._____.E.....	4.4 ± 1.2	18.8 ± 4.4
3Df (SEQ ID NO:26)	....._____A.....	1.6 ± 0.1	7.7 ± 0.4
3Dg (SEQ ID NO:27)	....._____..CC.G.ACTG..	6.1 ± 1.5	18.6 ± 1.5
3M (SEQ ID NO:28)	TCCATGTCGGTCCTGATGCT	4.1 ± 0.2	23.2 ± 4.9
3Ma (SEQ ID NO:29)	......CT.....	0.9 ± 0.1	1.8 ± 0.5
3Mb (SEQ ID NO:30)	.......E.....	1.3 ± 0.3	1.5 ± 0.6
3Mc (SEQ ID NO:31)	....._____..E.....	5.4 ± 1.5	8.5 ± 2.6
3Md (SEQ ID NO:7)	......A__T.....	17.2 ± 9.4	ND
3Me (SEQ ID NO:93)	....._____.....C..A.	3.6 ± 0.2	14.2 ± 5.2
4 (SEQ ID NO:94)	TCAACGTT	6.1 ± 1.4	19.2 ± 5.2
4a (SEQ ID NO:95)	....GC..	1.1 ± 0.2	1.5 ± 1.1
4b (SEQ ID NO:96)	...GCGC.	4.5.± 0.2	9.6 ± 3.4
4c (SEQ ID NO:97)	...TCGA.	2.7.± 1.0	ND
4d (SEQ ID NO:98)	..TT__AA	1.3 ± 0.2	ND
4e (Residue 2-8 of SEQ ID NO:94)	-..._____..	1.3 ± 0.2	1.1 ± 0.5
4f (SEQ ID NO:99)	C..._____..	3.9 ± 1.4	ND
4g (Residue 11-18 of SEQ ID NO:19)	--._____..CT	1.4 ± 0.3	ND
4h (SEQ ID NO:100)	...._____.C	1.2 ± 0.2	ND
LPS		7.8 ± 2.5	4.8 ± 1.0

'Stimulation indexes are the means and std. dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN.
ND = not done.
CpG dinucleotides are underlined.
Dots indicate identity; dashes indicate deletions.
E indicates 5 methyl cytosine.

TABLE 2

Identification of the optimal CpG motif for Murine IL-6 production and B cell activation

ODN	SEQUENCE (5'-3')	IL-6 (pg/ml)[a] CH12.LX	SPLENIC B CELL	SI[b]	IgM (ng/ml)[c]
512	(SEQ ID No:28) TCCATGTCGGTCCTGATGCT	1300 ± 106	627 ± 43	5.8 ± 0.3	7315 ± 1324
1637	(SEQ ID No:29) ......C.....	136 ± 27	46 ± 6	1.7 ± 0.2	770 ± 72
1615	(SEQ ID No:101) ......G.....	1201 ± 155	850 ± 202	3.7 ± 0.3	3212 ± 617
1614	(SEQ ID No:102) ......A.....	1533 ± 321	1812 ± 103	10.8 ± 0.6	7558 ± 414
1636	(SEQ ID No:103) .....A.....	1181 ± 76	947 ± 132	5.4 ± 0.4	3983 ± 485
1634	(SEQ ID NO:104) .....C.....	1049 ± 223	1671 ± 175	9.2 ± 0.9	6256 ± 261
1619	(SEQ ID No:105) .....T.....	1555 ± 304	2908 ± 129	12.5 ± 1.0	8243 ± 698
1618	(SEQ ID No:7) ......A..T.....	2109 ± 291	2596 ± 166	12.9 ± 0.7	10425 ± 674
1639	(SEQ ID No:3) .....AA..T.....	1827 ± 83	2012 ± 132	11.5 ± 0.4	9489 ± 103
1707	(SEQ ID No:88) ......A..TC.....	ND	1147 ± 175	4.0 ± 0.2	3534 ± 217
1708	(SEQ ID No:106) .....CA..TG.....	ND	59 ± 3	1.5 ± 0.1	466 ± 109

Dots indicate identity; CpG dinucleotides are underlined; ND = not done
[a]The experiment was done at least three times with similar results. The level of IL-6 of unstimulated control cultures of both CH12.LX and splenic B cells was ≤ 10 pg/ml. The IgM level of unstimulated culture was 547 ± 82 ng/ml. CpG dinucleotides are underlined and dots indicate identity.
[b][^{3}H] Uridine uptake was indicated as a fold increase (SI: stimulation index) from unstimulated control (2322.67 ± 213.68 cpm). Cells were stimulated with 20 μM of various CpG O-ODN. Data present the mean ± SD of triplicates
[c]Measured by ELISA.

The kinetics of lymphocyte activation were investigated using mouse spleen cells. When the cells were pulsed at the same time as ODN addition and harvested just four hours later, there was already a two-fold increase in ^{3}H uridine incorporation. Stimulation peaked at 12–48 hours and then decreased. After 24 hours, no intact ODN were detected, perhaps accounting for the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with CpG ODN, proliferation was found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude of stimulation was concentration dependent and consistently exceeded that of LPS under optimal conditions for both. Oligonucleotides containing a nuclease resistant phosphorothioate backbone were approximately two hundred times more potent than unmodified oligonucleotides.

Cell cycle analysis was used to determine the proportion of B cells activated by CpG-ODN. CpG-ODN induced cycling in more than 95% of B cells. Splenic B lymphocytes sorted by flow cytometry into CD23– (marginal zone) and CD23+ (follicular) subpopulations were equally responsive to ODN– induced stimulation, as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that CpG-ODN induce essentially all B cells to enter the cell cycle.

Immunostimulatory Nucleic Acid Molecules Block Murine B Cell Apoptosis

Certain B cell lines, such as WEHI-231, are induced to undergo growth arrest and/or apoptosis in response to crosslinking of their antigen receptor by anti-IgM (Jakway, J. P. et al., "Growth regulation of the B lymphoma cell line WEHI-231 by anti immunoglobulin, lipopolysaccharide and other bacterial products" *J. Immunol.* 137: 2225 (1986); Tsubata, T., J. Wu and T. Honjo: B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signal through CD40." *Nature* 364: 645 (1993)). WEHI-231 cells are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the CpG motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect. Subsequent work indicates that CpG ODN induce Bcl-x and myc expression, which may account for the protection from apoptosis. Also, CpG nucleic acids have been found to block apoptosis in human cells. This inhibition of apoptosis is important, since it should enhance and prolong immune activation by CpG DNA.

Identification of the optimal CpG motif for induction of Murine IL-6 and IgM secretion and B cell proliferation

To evaluate whether the optimal B cell stimulatory CpG motif was identical with the optimal CpG motif for IL-6 secretion, a panel of ODN in which the bases flanking the CpG dinucleotide were progressively substituted was studied. This ODN panel was analyzed for effects on B cell proliferation, Ig production, and IL-6 secretion, using both splenic B cells and CH12.LX cells. As shown in Table 2, the optimal stimulatory motif contains an unmethylated CpG flanked by two 5' purines and two 3' pyrimidines. Generally a mutation of either 5' purine to pyrimidine or 3' pyrimidine to purine significantly reduced its effects. Changes in 5' purines to C were especially deleterious, but changes in 5' purines to T or 3' pyrimidines to purines had less marked effects. Based on analyses of these and scores of other ODN, it was determined that the optimal CpG motif for induction

of IL-6 secretion is TGACGTT, which is identical with the optimal mitogenic and IgM-inducing CpG motif (Table 2). This motif was more stimulatory than any of the palindrome containing sequences studied (1639, 1707 and 1708).

Induction of Murine Cytokine Secretion by CpG motifs in Bacterial DNA or Oligonucleotides

As described in Example 9, the amount of IL-6 secreted by spleen cells after CpG DNA stimulation was measured by ELISA. T cell depleted spleen cell cultures rather than whole spleen cells were used for in vitro studies following preliminary studies showing, that T cells contribute little or nothing to the IL-6 produced by CpG DNA-stimulated spleen cells. As shown in Table 3, IL-6 production was markedly increased in cells cultured with *E. coli* DNA but not in cells cultured with calf thymus DNA. To confirm that the increased IL-6 production observed with *E. coli* DNA was not due to contamination by other bacterial products, the DNA was digested with DNAse prior to analysis. DNAse pretreatment abolished IL-6 production induced by *E. coli* DNA (Table 3). In addition, spleen cells from LPS-nonresponseive C3H/HeJ mouse produced similar levels of IL-6 in response to bacterial DNA. To analyze whether the IL-6 secretion induced by *E. coli* DNA was mediated by the unmethylated CpG dinucleotides in bacterial DNA, methylated *E. coli* DNA and a panel of synthetic ODN were examined. As shown in Table 3, CpG ODN significantly induced IL-6 secretion (ODN 5a, 5b, 5c) while CpG methylated *E. coli* DNA, or ODN containing methylated CpG (ODN 5f) or no CpG (ODN 5d) did not. Changes at sites other than CpG dinucleotides (ODN 5b) or methylation of other cytosines (ODN 5g) did not reduce the effect of CpG ODN. Methylation of a single CpG in an ODN with three CpGs resulted in a partial reduction in the stimulation (compare ODN 5c to 5e; Table 3).

TABLE 3

Induction of Murine IL-6 secretion by CpG motifs in bacterial DNA or oligonucleotides.

Treatment		IL-6 (pg/ml)
calf thymus DNA		≦10
calf thymus DNA + DNase		≦10
E. coli DNA		1169.5 ± 94.1
E. coli DNA + DNase		≦10
CpG methylated *E. coli* DNA		≦10
LPS		280.1 ± 17.1
Media (no DNA)		≦10
ODN		
5a	SEQ. ID. No:1 ATGGACTCTCCAGCGTTCTC	1096.4 ± 372.0
5b	SEQ. ID. No:2 .....AGG....A.....	1124.5 ± 126.2
5c	SEQ. ID. No:3 ..C.....G.....	1783.0 ± 189.5
5d	SEQ. ID. No:4 .... AGG..C..T.....	≦10
5e	SEQ. ID. No:5 ..C.....G..Z.....	851.1 ± 114.4
5f	SEQ. ID. No:6 ..Z.....ZG..Z.....	≦10
5g	SEQ. ID. No:7 ..C.....G.....Z..	1862.3 ± 87.26

T cell depleted spleen cells from DBA/2 mice were stimulated with phosphodiester modified oligonucleotides (O-ODN) (20 μM), calf thymus DNA (50 μg/ml) or *E. Coli* DNA (50 μg/ml) with or without enzyme treatment, or LPS (10 μg/ml) for 24 hr. Data represent the mean (pg/ml) ± SD of triplicates. CpG dinucleotides are underlined and dots indicate identity. Z indicates 5-methylcytosine.

CpG motifs can be used as an artificial adjuvant

Nonspecific simulators of the immune response are known as adjuvants. The use of adjuvants is essential to induce a strong antibody response to soluble antigens (Harlow and *Lane, Antibodies: A Laboratory manual,* Cold Spring harbor, N.Y. Current Edition; hereby incorporated by reference). The overall effect of adjuvants is dramatic and their importance cannot be overemphasized. The action of an adjuvant allows much smaller doses of antigen to be used and generates antibody responses that are more persistent. The nonspecific activation of the immune response often can spell the difference between success and failure in obtaining an immune response. Adjuvants should be used for first injections unless there is some very specific reason to avoid this. Most adjuvants incorporate two components. One component is designed to protect the antigen from rapid catabolism (e.g., liposomes or synthetic surfactants (Hunter et al. 1981)). Liposomes are only effective when the immunogen is incorporated into the outer lipid layer; entrapped molecules are not seen by the immune system. The other component is a substance that will stimulate the immune response nonspecifically. These substances act by raising the level of lymphokines. Lymphokines stimulate the activity of antigen-processing cells directly and cause a local inflammatory reaction at the site of injection. Early work relied entirely on heat-killed bacteria (Dienes 1936) or lipopolysaccaride (LPS) (Johnson et al. 1956). LPS is reasonably toxic, and, through analysis of its structural components, most of its properties as an adjuvant have been shown to be in a portion known as lipid A. Lipid A is available in a number of synthetic and natural forms that are much less toxic than LPS, but still retains most of the better adjuvant properties of parental LPS molecule. Lipid A compounds are often delivered using liposomes.

Recently an intense drive to find potent adjuvants with more acceptable side effects has led to the production of new synthetic adjuvants. The present invention provides the sequence 1826 TCCATGACGTTCCTGACGTT (SEQ ID NO: 10), which is an adjuvant including CpG containing nucleic acids. The sequence is a strong immune activating; sequence and is a superb adjuvant, with efficacy comparable or superior to complete Freund's, but without apparent toxicity.

Titration of induction of Murine IL-6 Secretion by CpG motifs

Bacterial DNA and CpG ODN induced IL-6 production in T cell depleted murine spleen cells in a dose-dependent

manner, but vertebrate DNA and non-CpG ODN did not (FIG. 1). IL-6 production plateaued at approximately 50 μg/ml of bacterial DNA or 40 μM of CpG O-ODN. The maximum levels of IL-6 induced by bacterial DNA and CpG ODN were 1–1.5 ng/ml and 2–4 ng/ml respectively. These levels were significantly greater than those seen after stimulation by LPS (0.35 ng/ml) (FIG. 1A). To evaluate whether CpG ODN with a nuclease-resistant DNA backbone would also induce IL-6 production, S-ODN were added to T cell depleted murine spleen cells. CpG S-ODN also induced IL-6 production in a dose-dependent manner to approximately the same level as CpG O-ODN while non-CpG S-ODN failed to induce IL-6 (FIG. 1C). CpG S-ODN at a concentration of 0.05 μM could induce maximal IL-6 production in these cells. This result indicated that the nuclease-resistant DNA backbone modification retains the sequence specific ability of CpG DNA to induce IL-6 secretion and that CpG S-ODN are more than 80-fold more potent than CpG O-ODN in this assay system.

Induction of Murine IL-6 secretion by CpG DNA in vivo

To evaluate the ability of bacterial DNA and CpG S-ODN to induce IL-6 secretion in vivo, BALB/c mice were injected iv. with 100 μg of *E. coli* DNA, calf thymus DNA, or CpG or non-stimulatory S-ODN and bled 2 hr after stimulation. The level of IL-6 in the sera from the *E. coli* DNA injected group was approximately 13 ng/ml while IL-6 was not detected in the sera from calf thymus DNA or PBS injected groups (Table 4). CpG S-ODN also induced IL-6 secretion in vivo. The IL-6 level in the sera from CpG S-ODN injected groups was approximately 20 ng/mL. In contrast, IL-6 was not detected in the sera from non-stimulatory S-ODN stimulated group (Table 4).

TABLE 4

Secretion of Murine IL-6 induced by CpG DNA stimulation in vivo.

Stimulant	IL-6 (pg/ml)
PBS	<50
E. coli DNA	13858 ± 3143
Calf Thymus DNA	<50
CpG S-ODN	20715 ± 606
non-CpG S-ODN	<50

Mice (2 mice/group) were i.v. injected with 100 μl of PBS, 200 μg of *E. coli* DNA or calf thymus DNA, or 500 μg of CpG S-ODN or non-CpG control S-ODN. Mice were bled 2 hr after injection and 1:10 dilution of each serum was analyzed by IL-6 ELISA. Sensitivity limit of IL-6 ELISA was 5 pg/ml. Sequences of the CpG S-ODN is 5'GCATGACGT-TGAGCT3' (SEQ. ID. No: 6) and of the non-stimulatory S-ODN is 5'GCTAGATGTTAGCGT3' (SEQ. ID. No: 49). Note that although there is a CpG in sequence 48, it is too close to the 3' end to effect stimulation, as explained herein. Data represent mean ± SD of duplicates. The experiment was done at least twice with similar results.

Kinetics of Murine IL-6 secretion after stimulation by CpG motifs in vivo

To evaluate the kinetics of induction of IL-6 secretion by CpG DNA in vivo, BALB/c mice were injected iv. with CpG or control non-CpG S-ODN. Serum IL-6 levels were significantly increased within 1 hr and peaked at 2 hr to a level of approximately 9 ng/ml in the CpG S-ODN injected group (FIG. 2). IL-6 protein in sera rapidly decreased after 4 hr and returned to basal level by 12 hr after stimulation. In contrast to CpG DNA stimulated groups, no significant increase of IL-6 was observed in the sera from the non-stimulatory S-ODN or PBS injected groups (FIG. 2).

Tissue distribution and kinetics of IL-6 mRNA expression induced by CpG moths in vivo

As shown in FIG. 2, the level of serum IL-6 increased rapidly after CpG DNA stimulation. To investigate the possible tissue origin of this serum IL-6, and the kinetics of IL-6 gene expression in vivo after CpG DNA stimulation, BALB/c mice were injected iv with CpG or non-CpG S-ODN and RNA was extracted from liver, spleen, thymus, and bone marrow at various time points after stimulation. As shown in FIG. 3A, the level of IL-6 mRNA in liver, spleen, and thymus was increased within 30 min. after injection of CpG S-ODN. The liver IL-6 mRNA peaked at 2 hr post-injection and rapidly decreased and reached basal level 8 hr after stimulation (FIG. 3A). Splenic IL-6 mRNA peaked at 2 hr after stimulation and then gradually decreased (FIG. 3A). Thymus IL-6 mRNA peaked at 1 hr post-injection and then gradually decreased (FIG. 3A). IL-6 mRNA was significantly increased in bone marrow within 1 hr after CpG S-ODN injection but then returned to basal level. In response to CpG S-ODN, liver, spleen and thymus showed more substantial increases in IL-6 mRNA expression than the bone marrow.

Patterns of Murine Cytokine Expression Induced by CpG DNA

In vivo or in whole spleen cells, no significant increase in the protein levels of the following interleukins: IL-2, IL-3, IL-4, IL-5, or IL-10 was detected within the first six hours (Klinman, D. M. et al., (1996) *Proc. Natl. Acad. Sci. USA* 93:2879–2883). However, the level of TNF-α is increased within 30 minutes and the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with CpG ODN. Increased expression of IL-12 and interferon gamma (IFN-γ) mRNA by spleen cells was also detected within the first two hours.

TABLE 5

Induction of human PBMC cytokine secretion by CpG oligos

ODN	Sequence (5'–3')	IL-6,	TNF-α1	IFN-γ1	GM-CSF	IL-12
512 SEQ ID NO:28	TCCATGTCGGTCCTGATGCT	500	140	15.6	70	250
1637 SEQ ID NO:29	----C----------	550	16	7.8	15.6	16
1615 SEQ ID NO:101	----G----------	600	145	7.8	45	145

TABLE 5-continued

Induction of human PBMC cytokine secretion by CpG oligos

ODN	Sequence (5'–3')	IL-6[1]	TNF-α[1]	IFN-γ[1]	GM-CSF	IL-12
1614 SEQ ID NO:102	.....A$_{CG}$.....	550	31	0	50	31
1636 SEQ ID NO:103	.....A.....	325	250	35	40	250
1634 SEQ ID NO:104	.....C.....	300	400	40	85	400
1619 SEQ ID NO:105	.....T.....	275	450	200	80	450
1618 SEQ ID NO:7	.....A$_{CG}$T.....	300	60	15.6	15.6	62
1639 SEQ ID NO:3	....AA$_{CG}$T.....	625	220	15.6	40	220
1707 SEQ ID NO:88	.....A$_{CG}$TC.....	300	70	17	0	70
1708 SEQ ID NO:106	....CA$_{CG}$TG.....	270	10	17	ND	10

dots indicate identity; CpG dinucleotides are underlined

[1]measured by ELISA using Quantikine kits from R&D Systems (pg/ml) Cells were cultured in 10% autologous serum with the indicated oligodeoxynucleotides (12 μg/ml) for 4 hr in the case of TNF-α or 24 hr for the other cytokines before supernatant harvest and assay. Data are presented as the level of cytokine above that in wells with no added oligodeoxynucleotide.

CpG DNA induces cytokine secretion by human PBMC, specifically monocytes

The same panels of ODN used for studying mouse cytokine expression were used to determine whether human cells also are induced by CpG motifs to express cytokine (or proliferate), and to identify the CpG motif(s) responsible. Oligonucleotide 1619 (GTCGTT; residues 6–11 of SEQ ID NO:105) was the best inducer of TNF-$\alpha$and IFN-γ secretion, and was closely followed by a nearly identical motif in oligonucleotide 1634 (GTCGCT; residues 6–11 of SEQ ID NO:104) (Table 5). The motifs in oligodeoxynucleotides 1637 and 1614 (GCCGGT; residues of SEQ ID NO:29 and GACGGT; residues 6–11 of SEQ ID NO:102) led to strong IL-6 secretion with relatively little induction of other cytokines. Thus, it appears that human lymphocytes, like murine lymphocytes, secrete cytokines differentially in response to CpG dinucleotides, depending on the surrounding bases. Moreover, the motifs that stimulate murine cells best differ from those that are most effective with human cells. Certain CpG oligodeoxynucleotides are poor at activating human cells (oligodeoxynucleotides 1707, 1708, which contain the palindrome forming sequences GACGTC; residues 6–11 of SEQ ID NO:8 and CACGTG; residues 6–11 of SEQ ID NO:106 respectively).

The cells responding to the DNA appear to be monocytes, since the cytokine secretion is abolished by treatment of the cells with L-leucyl-L-leucine methyl ester (L-LME), which is selectively toxic to monocytes (but also to cytotoxic T lymphocytes and NK cells), and does not affect B cell Ig secretion (Table 6). The cells surviving L-LME treatment had >95% viability by trypan blue exclusion, indicating that the lack of a cytokine response among these cells did not simply reflect a nonspecific death of all cell types Cytokine secretion in response to *E. coli* (EC) DNA requires unmethylated CpG motifs, since it is abolished by methylation of the EC DNA (next to the bottom row, Table 6). LPS contamination of the DNA cannot explain the results since the level of contamination was identical in the native and methylated DNA, and since addition of twice the highest amount of contaminating LPS had no effect (not shown).

TABLE 6

CpG DNA induces cytokine secretion by human PBMC

DNA	TNF-α(pg/ml)[1]	IL-6 (pg/ml)	IFN-γ (pg/ml)	RANTES (pg/ml)
EC DNA (50 μg/ml)	900	12,000	700	1560
EC DNA (5 μg/ml)	850	11,000	400	750
EC DNA (0.5 μ/ml)	500	ND	200	0
EC DNA (0.05 μg/ml)	62.5	10,000	15.6	0
EC DNA (50 μg/ml) + L-LME[2]	0	ND	ND	ND
EC DNA (10 μg/ml) Methyl[3]	0	5	ND	ND
CT DNA (50 μg/ml)	0	600	0	0

[1]Levels of all cytokines were determined by ELISA using Quantikine kits from R&D Systems as described in the previous table. Results are representative using PBMC from different donors.
[2]Cells were pretreated for 15 min. with L-leucyl-L-leucine methyl ester (M-LME) to determine whether the cytokine production under these conditions was from monocytes (or other L-LME-sensitive cells).
[3]EC DNA was methylated using 2U/μg DNA of CpG methylase (New England Biolabs) according to the manufacturer's directions, and methylation confirmed by digestion with Hpa-II and Msp-I. As a negative control, samples were included containing twice the maximal amount of LPS contained in the highest concentration of EC DNA which failed to induce detectable cytokine production under these experimental conditions.
ND = not done

The loss of cytokine production in the PBMC treated with L-LME suggested that monocytes may be responsible for cytokine production in response to CpG DNA. To test this hypothesis more directly, the effects of CpG DNA on highly purified human, monocytes and macrophages was tested. As hypothesized, CpG DNA directly activated production of the cytokines IL-6, GM-CSF, and TNF-α by human macrophages, whereas non-CpG DNA did not (Table 7).

TABLE 7

CpG DNA induces cytokine expression in purified human macrophages

	IL-6 (pg/ml)	GM-CSF (pg/ml)	TNF-α(pg/ml)
Cells alone	0	0	0
CT DNA (50 μg/ml)	0	0	0
EC DNA (50 μg/ml)	2000	15.6	1000

Biological Role of IL-6 in Inducing Murine IgM Production in Response to CpG Motifs

The kinetic studies described above revealed that induction of IL-6 secretion, which occurs within 1 hr post CpG stimulation, precedes IgM secretion. Since the optimal CpG motif for ODN inducing secretion of IL-6 is the same as that for IgM (Table 2), whether the CpG motifs independently induce IgM and IL-6 production or whether the IgM production is dependent on prior IL-6 secretion was examined. The addition of neutralizing anti-IL-6 antibodies inhibited in vitro IgM production mediated by CpG ODN in a dose-dependent manner but a control antibody did not (FIG. 4A). In contrast, anti-IL-6 addition did not affect either the basal level or the CpG-induced B cell proliferation (FIG. 4B).

Increased transcriptional activity of the IL-6promoter in response to CpG DNA

The increased level of IL-6 mRNA and protein after CpG DNA stimulation could result from transcriptional or post-transcriptional regulation. To determine if the transcriptional activity of the IL-6 promoter was upregulated in B cells cultured with CpG ODN, a murine B cell line, WEHI-231, which produces IL-6 in response to CpG DNA, was transfected with an IL-6 promoter-CAT construct (pIL-6/CAT) (Pottratz, S. T. et al., 17B-estradiol) inhibits expression of human interleukin-6-promoter-reporter constructs by a receptor-dependent mechanism. *J. Clin. Invest.* 93:944). CAT assays were performed after stimulation with various concentrations of CpG or non-CpG ODN. As shown in FIG. 5, CpG ODN induced increased CAT activity in dose-dependent manner while non-CpG ODN failed to induce CAT activity. This confirms that CpG induces the transcriptional activity of the IL-6 promoter.

Dependence of B cell activation by CpG ODN on the Number of 5' and 3' Phosphorothioate Internucleotide Linkages

To determine whether partial sulfur modification of the ODN backbone would be sufficient to enhance B cell activation, the effects of a series of ODN with the same sequence, but with differing numbers of S internucleotide linkages at the 5' and 3' ends were tested. Based on previous studies of nuclease degradation of ODN, it was determined that at least two phosphorothioate linkages at the 5' end of ODN were required to provide optimal protection of the ODN from degradation by intracellular exo- and endonucleases. Only chimeric ODN containing two 5' phosphorothioate-modified linkages, and a variable number of 3' modified linkages were therefore examined.

The lymphocyte stimulating effects of these ODN were tested at three concentrations (3.3, 10, and 30 μM) by measuring the total levels of RNA synthesis (by ^{3}H uridine incorporation) or DNA synthesis (by ^{3}H thymidine incorporation) in treated spleen cell cultures (Example 10). O-ODN (0/0 phosphorothioate modifications) bearing a CpG motif caused no spleen cell stimulation unless added to the cultures at concentrations of at least 10 μM (Example 10). However, when this sequence was modified with two S linkages at the 5' end and at least three S linkages at the 3' end, significant stimulation was seen at a dose of 3.3 μM. At this low dose, the level of stimulation showed a progressive increase as the number of 3' modified bases was increased, until this reached or exceeded six, at which point the stimulation index began to decline. In general, the optimal number of 3' S linkages for spleen cell stimulation was five. Of all three concentrations tested in these experiments, the S-ODN was less stimulatory than the optimal chimeric compounds.

Dependence of CpG-mediated lymphocyte activation on the type of backbone modification

Phosphorothioate modified ODN (S-ODN) are far more nuclease resistant than phosphodiester modified ODN (O-ODN). Thus, the increased immune stimulation caused by S-ODN and S-O-ODN (i.e., chimeric phosphorothioate ODN in which the central linkages are phosphodiester, but the two 5' and five 3' linkages are phosphorothioate modified) compared to O-ODN may result from the nuclease resistance of the former. To determine the role of ODN nuclease resistance in immune stimulation by CpG ODN, the stimulatory effects of chimeric ODN in which the 5' and 3' ends were rendered nuclease resistant with either methylphosphonate (MP-), methylphosphorothioate (MPS-), phosphorothioate (S-), or phosphorodithioate (S_2-) internucleotide linkages were tested (Example 10). These studies showed that despite their nuclease resistance, MP-O-ODN were actually less immune stimulatory than O-ODN. However, combining the MP and S modifications by replacing both nonbridging O molecules with 5' and 3' MPS internucleotide linkages restored immune stimulation to a slightly higher level than that triggered by O-ODN.

S-O-ODN were far more stimulatory than O-ODN, and were even more stimulatory than S-ODN, at least at concentrations above 3.3 μM. At concentrations below 3 μM, the S-ODN with the 3M sequence was more potent than the corresponding S-O-ODN, while the S-ODN with the 3D sequence was less potent than the corresponding S-O-ODN (Example 10). In comparing the stimulatory CpG motifs of these two sequences, it was noted that the 3D sequence is a perfect match for the stimulatory motif in that the CpG is flanked by two 5' purines and two 3' pyrimidines. However, the bases immediately flanking the CpG in ODN 3D are not optimal; it has a 5' pyrimidine and a 3' purine. Based on further testing, it was found that the sequence requirement for immune stimulation is more stringent for S-ODN than for S-O- or O-ODN. S-ODN with poor matches to the optimal CpG motif cause little or no lymphocyte activation (e.g. Sequence 3D). However, S-ODN with good matches to the motif, most critically at the positions immediately flanking the CpG, are more potent than the corresponding S-O-ODN (e.g. Sequence 3M, Sequences 4 and 6), even though at higher concentrations (greater than 3 μM) the peak effect from the S-O-ODN is greater (Example 10).

S_2-O-ODN were remarkably stimulatory, and caused substantially greater lymphocyte activation than the corresponding S-ODN or S-O-ODN at every tested concentration.

The increased B cell stimulation seen with CpG ODN bearing S or S_2 substitutions could result from any or All of the following effects: nuclease resistance, increased cellular uptake, increased protein binding, and altered intracellular localization. However, nuclease resistance can not be the only explanation, since the MP-O-ODN were actually less stimulatory than the O-ODN with CpG motifs. Prior studies have shown that ODN uptake by lymphocytes is markedly affected by the backbone chemistry (Zhao et al., (1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides.

(Antisense Research and Development 3, 53–66; Zhao et al., (1994) Stage specific oligonucleotide uptake in murine bone marrow B cell precursors. Blood 84, 3660–3666.) The highest cell membrane binding and uptake was seen with S-ODN, followed by S-O-ODN, O-ODN, and MP-ODN. This differential uptake correlates well with the degree of immune stimulation.

Unmethylated CpG Containing Oligos Have NK Cell Stimulatory Activity

Experiments were conducted to determine whether CpG containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 8, a marked induction of NK activity among spleen cells cultured with CpG ODN 1 and 3Dd was observed. In contrast, there was relatively no induction in effectors that had been treated with non-CpG control ODN.

TABLE 8

Induction Of NK Activity By CpG Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis* Effector: Target		% 2C11 Specific Lysis Effector: Target	
ODN	50:1	100:1	50:1	100:1
None	−1.1	−1.4	15.3	16.6
1	16.1	24.5	38.7	47.2
3Dd	17.1	27.0	37.0	40.0
non-CpG ODN	−1.6	−1.7	14.8	15.4

Induction of NK activity by DNA containing CpG motifs, but not by non-CpG DNA

Bacterial DNA cultured for 18 hrs. at 37° C. and then assayed for killing of K562 (human) or Yac-1 (mouse) target cells induced NK lytic activity in both mouse spleen cells depleted of B cells and human PBMC, but vertebrate DNA did not (Table 9). To determine whether the stimulatory activity of bacterial DNA may be a consequence of its increased level of unmethylated CpG dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no CpG dinucleotides was tested. The results, summarized in Table 9, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated CpG dinucleotide. No difference was observed in the stimulatory effects of ODN in which the CpG was within a palindrome (such as ODN 1585, which contains the palindrome AACGTT) from those ODN without palindromes (such as 1613 or 1619), with the caveat that optimal stimulation was generally seen with ODN in which the CpG was flanked by two 5' purines or a 5' GpT dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK activity peaked around 18 hrs. after addition of the ODN. The data indicates that the murine NK; response is dependent on the prior activation of monocytes by CpG DNA, leading to the production of IL-12, TNF-α, and IFN-α/b (Example 11).

TABLE 9

Induction of NK Activity by DNA Containing CpG Motifs but not by Non-CpG DNA

	DNA or Cytokine Added		LU/10⁶ Mouse Cells	LU/10⁶ Human Cells
Expt. 1	None		0.00	0.00
	IL-2		16.68	15.82
	E.Coli. DNA		7.23	5.05
	Calf thymus DNA		0.00	0.00
Expt. 2	None		0.00	3.28
	1585 ggGGTCAACGTTGACgggg	(SEQ ID No.12)	7.38	17.98
	1629 -------gtc-----	(SEQ ID No.50)	0.00	4.4
Expt. 3	None		0.00	
	1613 GCTAGACGTTAGTGT	(SEQ ID No.42)	5.22	
	1769 -------Z-----	(SEQ ID No.52)	0.02	ND
	1619 TCCATGTCGTTCCTGATGCT	(SEQ ID No:38)	3.35	
	1765 -------Z---------	(SEQ ID No.53)	0.11	

CpG dinucleotides in ODN sequences are indicated by underlying; Z indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide linkages which, in titration experiments, were more than 20 times as potent as non-modified ODN, depending on the flanking bases. Poly G ends (g) were used in some ODN, because they significantly increase the level of ODN uptake.

From all of these studies, a more complete understanding of the immune, effects of CpG DNA has been developed, which is summarized in FIG. 6.

Immune activation by CpG motifs may depend on bases flanking the CpG, and the number and spacing of the CpGs present within an ODN. Although a single CpG in an ideal base context can be a very strong and useful immune activator, superior effects can be seen with ODN containing several CpGs with the appropriate spacing and flanking bases. For activation of murine B cells, the optimal CpG motif is TGACGTT; residues 10–17 of SEQ ID NO:70.

The following studies were conducted to identify optimal ODN sequences for stimulation of human cells by examining the effects of changing the number, spacing, and flanking bases of CpG dinucleotides.

Identification of phosphorothioate ODN with optimal CpG motifs for activation of human NK cells

To have clinical utility, ODN must be administered to a subject in a form that protects them against nuclease degradation. Methods to accomplish this with phosphodiester ODN are well known in the art and include encapsulation in lipids or delivery systems such as nanoparticles. This protection can also be achieved using chemical substitutions to the DNA such as modified DNA backbones including those in which the internucleotide linkages are nuclease resistant. Some modifications may confer additional desirable properties such as increasing cellular uptake. For example, the phosphodiester linkage can be modified via replacement of one of the nonbridging oxygen atoms with a sulfur, which constitutes phosphorothioate DNA. Phosphorothioate ODN have enhanced cellular uptake (Krieg et al., Antisense Res. Dev. 6:133, 1996.) and improved B cell stimulation if they also have a CpG motif. Since NK activation correlates strongly with in vivo adjuvant effects, the identification of phosphorothioate ODN that will activate human NK cells is very important.

The effects of different phosphorothioate ODNs—containing CpG dinucleotides in various base contexts—on human NK activation (Table 10) were examined. ODN 1840, which contained 2 copies of the TGTCGTT motif (residues 14–20 of SEQ ID NO:47), had significant NK lytic activity (Table 10). To further identify additional ODNs optimal for NK activation, approximately one hundred ODN containing different numbers and spacing of CpG motifs, were tested with ODN 1982 serving as a control. The results are shown in Table 11.

Effective ODNs began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal CpG motifs (e.g. ODN 1840) are generally less potent stimulators than those in which a GTCGCT motif (residues 3–8 of SEQ ID NO:54) immediately follows the 5' TC (e.g., ODN 1967 and 1968). ODN 1968, which has a second GTCGTT motif (residues 3–8 of SEQ ID NO:46) in its 3' half, was consistently more stimulatory than ODN 1967, which lacks this second motif. ODN 1967, however, was slightly more potent than ODN 1968 in experiments 1 and 3, but not in experiment 2. ODN 2005, which has a third GTCGTT motif (residues 3–8 of SEQ ID NO:46) induced slightly higher NK activity on average than 1968. However, ODN 2006, in which the spacing between the GTCGTT motifs (residues 3–8 of SEQ ID NO:46) was increased by the addition of two Ts between each motif, was superior to ODN 2005 and to ODN 2007, in which only one of the motifs had the addition of the spacing two Ts. The minimal acceptable spacing between CpG motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN 2015). Surprisingly, joining two GTCGTT motifs (residues 3–8 of SEQ ID NO:46) end to end with a 5' T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The choice of thymine (T) separating consecutive CpG dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (i.e., CGACGTT; residues 14–20 of SEQ ID NO:82). It should also be noted that ODNs containing no CpG (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contexts (e.g., ODN 2010) had no stimulatory effect on NK activation.

TABLE 10

ODN induction of NK Lytic Activity (LU)

ODN cells alone	Sequence (5'–3')	LU 0.01	
1754	ACCATGGACGATCTGTTTCCCCTC	0.02	SEQ ID NO: 59
1758	TCTCCCAGCGTGCGCCAT	0.05	SEQ ID NO: 45
1761	TACCGCGTGCGACCCTCT	0.05	SEQ ID NO: 60
1776	ACCATGGACGAACTGTTTCCCCTC	0.03	SEQ ID NO: 61
1777	ACCATGGACGAGCTGTTTCCCCTC	0.05	SEQ ID NO: 62
1778	ACCATGGACGACCTGTTTCCCCTC	0.01	SEQ ID NO: 63
1779	ACCATGGACGTACTGTTTCCCCTC	0.02	SEQ ID NO: 64
1780	ACCATGGACGGTCTGTTTCCCCTC	0.29	SEQ ID NO: 65
1781	ACCATGGACGTTCTGTTTCCCCTC	0.38	SEQ ID NO: 66
1823	GCATGACGTTGAGCT	0.08	SEQ ID NO: 6
1824	CACGTTGAGGGGCAT	0.01	SEQ ID NO: 67
1825	CTGCTGAGACTGGAG	0.01	SEQ ID NO: 68
1828	TCAGCGTGCGCC	0.01	SEQ ID NO: 69
1829	ATGACGTTCCTGACGTT	0.42	SEQ ID NO: 70
1830[2]	RANDOM SEQUENCE	0.25	
1834	TCTCCCAGCGGGCGCAT	0.00	SEQ ID NO: 71
1836	TCTCCCAGCGCGCGCCAT	0.46	SEQ ID NO: 72
1840	TCCATGTCGTTCCTGTCGTT	2.70	SEQ ID NO: 73
1841	TCCATAGCGTTCCTAGCGTT	1.45	SEQ ID NO: 74
1842	TCGTCGCTGTCTCCGCTTCTT	0.06	SEQ ID NO: 75
1851	TCCTGACGTTCCTGACGTT	2.32	SEQ ID NO: 76

[1]Lytic units (LU) were measured as described (8). Briefly, PBMC were collected from normal donors and spun over Ficoll, then cultured with or without the indicated ODN (which were added to cultures at 6 μg/ml) for 24 hr. Then their ability to lyse ^{51}Cr-labeled K562 cells was determined. The results shown are typical of those obtained with several different normal human donors. [2]This oligo mixture contained a random selection of all 4 bases at each position.

TABLE 11

Induction of NK LU by Phoshorothioate CpG ODN with Good Motifs

ODN[1] cells alone	sequence (5'–3')	SEQ ID NO:	expt. 1 0.00	expt. 2 1.26	expt. 3 0.46
1840	TCCATGTCGTTCCTGTCGTT	73	2.33	ND	ND
1960	TCCTGTCGTTCCTGTCGTT	77	ND	0.48	8.99
1961	TCCATGTCGTTTTTGTCGTT	78	4.03	1.23	5.08
1962	TCCTGTCGTTCCTTGTCGTT	52	ND	1.60	5.74
1963	TCCTTGTCGTTCCTGTCGTT	79	3.42	ND	ND
1965	TCCTGTCGTTTTTTGTCGTT	53	0.46	0.42	3.48
1966	TCGTCGCTGTCTCCGCTTCTT	75	2.62	ND	ND
1967	TCGTCGCTGTCTGCCCTTCTT	54	5.82	1.64	8.32
1968	TCGTCGCTGTTGTCGTTTCTT	55	3.77	5.26	6.12
1979[2]	TCCATGTZGTTCCTGTZGTT		1.32	ND	ND
1982	TCCAGGACTTCTCTCAAGTT	79	0.05	ND	0.98
1990	TCCATGCGTGCGTGCGTTTT	80	2.10	ND	ND
1991	TCCATGCGTTGCGTTGCGTT	81	0.89	ND	ND
2002	TCCACGACGTTTTCGACGTT	82	4.02	1.31	9.79
2005	TCGTCGTTGTCGTTGTCGTT	47	ND	4.22	12.75
2006	TCGTCGTTTTGTCGTTTTGTCGT	56	ND	6.17	12.82
2007	TCGTCGTTGTCGTTTTGTCGTT	49	ND	2.68	9.66
2008	GCGTGCGTTGTCGTTGTCGTT	56	ND	1.37	8.15
2010	GCGGCGGGCGGCGCGCGCCC	83	ND	0.01	0.05
2012	TGTCGTTTGTCGTTTGTCGTT	48	ND	2.02	11.61
2013	TGTCGTTGTCGTTGTCGTTGTCGTT	84	ND	0.56	5.22
2014	TGTCGTTGTCGTTGTCGTT	60	ND	5.74	10.89
2015	TCGTCGTCGTCGTT	51	ND	4.53	10.13
2016	TGTCGTTGTCGTT	85	ND	6.54	8.06

[1]PBMC essentially as described herein. Results are representative of 6 separate experiments; each experiment represents a different donor. [2]This is the methylated version of ODN 1840 (SEQ ID NO:83); Z = 5-methyl cytosine at residues 8 and 17; LU is lytic units; ND = not done; CpG dinucleotides are underlined for clarity

Identification of phosphorothioate ODN with optimal CpG motifs for activation of human B cell proliferation

The ability of a CpG ODN to induce B cell proliferation is a good measure of its adjuvant potential. Indeed, ODN with strong adjuvant effects generally also induce B cell proliferation. To determine whether the optimal CpG ODN for inducing B cell proliferation are the same as those for inducing NK cell activity, similar panels of ODN (Table 12) were tested. The most consistent stimulation appeared with ODN 2006 (Table 12).

TABLE 12

Induction of human B cell proliferation by Phosphorothioate CpG ODN

ODN sequence (5' 3')	SEQ ID NO:	Stimulation Index[1L] expt. 1	expt. 2	expt. 3	expt. 4	expt. 5	expt. 6
1840 TCCATGTCGTTCCTGTCGTT	73	4	ND	ND	ND	ND	34
1841 TCCATAGCGTTCCTAGCGTT	74	3	ND	ND	ND	ND	ND

TABLE 12-continued

Induction of human B cell proliferation by Phosphorothioate CpG ODN

ODN sequence (5' 3')	SEQ ID NO:	Stimulation Index[1] expt. 1	expt. 2	expt. 3	expt. 4	expt. 5	expt. 6
1960 TCCTGTCGTTCCTGTCGTT	77	ND	2.0	2.0	3.6	ND	ND
1961 TCCATGTCGTTTTTGTCGTT	78	2	3.9	1.9	3.7	ND	37
1962 TCCTGTCGTTCCTTGTCGTT	52	ND	3.8	1.9	3.9	5.4	35
1963 TCCTTGTCGTTCCTGTCGTT	79	3	ND	ND	ND	ND	ND
1965 TCCTGTCGTTTTTTGTCGTT	53	4	3.7	2.4	4.7	6.0	43
1967 TCGTCGCTGTCTGCCCTTCTT	54	ND	4.4	2.0	4.5	5.0	36
1968 TCGTCGCTGTTGTCGTTTCTT	55	ND	4.0	2.0	4.9	8.7	38
1982 TCCAGGACTTCTCTCAGGTT	79	3	1.8	1.3	3.1	3.2	12
2002 TCCACGACGTTTTCGACGTT	86	ND	2.7	1.4	4.4	ND	14
2005 TCGTCGTTGTCGTTGTCGTT	47	5	3.2	1.2	3.0	7.9	37
2006 TCGTCGTTTTGTCGTTTTGTCGTT	46	4	4.5	2.2	5.8	8.3	40
2007 TCGTCGTTGTCGTTTTGTCGTT	49	3	4.0	4.2	4.1	ND	22
2008 GCGTGCGTTGTCGTTGTCGTT	56	ND	3.0	2.4	1.6	ND	12
2010 GCGGCGGGCGGCGCGCGCCC	83	ND	1.6	1.9	3.2	ND	ND
2012 TGTCGTTTGTCGTTTGTCGTT	48	2	2.8	0	3.2	ND	33
2013 TGTCGTTGTCGTTGTCGTTGTCGTT	84	3	2.3	3.1	2.8	ND	7
2014 TGTCGTTGTCGTTGTCGTT	50	3	2.5	4.0	3.2	6.7	14
2015 TCGTCGTCGTCGTT	51	5	1.8	2.6	4.5	9.4	1
2016 TGTCGTTGTCGTT	85	ND	1.1	1.7	2.7	7.3	1

[1]Cells = human spleen cells stored at -70° C. after surgical harvest or PBMC collected from normal donors and spun over Ficoll. Cells were cultured in 96 well U-bottom microtiter plates with or without the indicated ODN (which were added to cultures at 6 μml). N = 12 experiments. Cells were cultured for 4–7 days, pulsed with 1 μCi of ^{3}H thymidine for 18 hr before harvest and scintillation counting. Stimulation index = the ratio of cpm in wells without ODN to that in wells that had been stimulated throughout the culture period with the indicated ODN (there were no further additions of ODN after the cultures were set up). ND = not done

Identification of phosphorothioate ODN that induce human IL-12 secretion

The ability of a CpG ODN to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to induce a Th1 immune response, which is highly dependent on IL-12. Therefore, the ability of a panel of phosphorothioate ODN to induce IL-12 secretion from human PBMC in vitro (Table 13) was examined. These experiments showed that in some human PBMC, most CpG ODN could induce IL-12 secretion (e.g., expt. 1). However, other donors responded to just a few CpG ODN (e.g., expt. 2). ODN 2006 was a consistent inducer of IL2 secretion from most subjects (Table 13).

TABLE 13

Induction of human IL-12 secretion by Phosphorothioate CpG ODN

ODN[1]	sequence (5'–3')	SEQ ID NO	IL-12 (pg/ml) expt. 1	expt. 2
cells alone			0	0
1962	TCCTGTCGTTCCTTGTCGTT	52	19	0
1965	TCCTGTCGTTTTTTGTCGTT	53	36	0
1967	TCGTCGCTGTCTGCCCTTCTT	54	41	0
1968	TCGTCGCTGTTGTCGTTTCTT	55	24	0
2005	TCGTCGTTGTCGTTGTCGTT	47	25	0
2006	TCGTCGTTTTGTCGTTTTGTCGTT	46	29	15

TABLE 13-continued

Induction of human IL-12 secretion by Phosphorothioate CpG ODN

ODN[1]	sequence (5'-3')	SEQ ID NO	IL-12 (pg/ml) expt. 1	IL-12 (pg/ml) expt. 2
2014	TGTCGTTGTCGTTGTCGTT	50	28	0
2015	TCGTCGTCGTCGTT	51	14	0
2016	TGTCGTTGTCGTT	85	3	0

[1]PBMC were collected from normal donors and spun over Ficoll, then cultured at 10^6 cells/well in 96 well microtiter plates with or without the indicated ODN which were added to cultures at 6 μg/ml. Supernatants were collected at 24 hr and tested for IL-12 levels by ELISA as described in methods. A standard curve was run in each experiment, which represents a different donor.

Identification of B cell and monocyte/NK cell-specific oligonucleotides

As shown in FIG. 6, CpG DNA can directly activate highly purified B cells and monocytic cells. There are many similarities in the mechanism through which CpG DNA activates these cell types. For example, both require NFkB activation as explained further below.

In further studies of different immune effects of CpG DNA, it was found that there is more than one type of CpG motif. Specifically, oligo 1668, with the best mouse B cell motif, is a strong inducer of both B cell and natural killer (NK) cell activation, while oligo 1758 is a weak B cell activator, but still induces excellent NK responses (Table 14).

TABLE 14

Different CpG motifs stimulate optimal murine B cell and NK activation

ODN	Sequence		B cell activation[1]	NK activation[2]
1668	TCCATGACGTTCCTGATGCT	(SEQ.ID.NO:7)	42,849	2.52
1758	TCTCCCAGCGTGCGCCAT	(SEQ.ID.NO.45)	1,747	6.66
NONE			367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance. [1]Measured by ^{3}H thymidine incorporation after 48 hr culture with oligodeoxynucleotides at a 200 nM concentration as described in Example 1. [2]Measured in lytic units.

Teleological Basis of Immunostimulatory, Nucleic Acids

Vertebrate DNA is highly methylated and CpG dinucleotides are underrepresented. However, the stimulatory CpG motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported to induce B cell proliferation 2nd immunoglobulin (Ig) production, while mammalian DNA does not (Messina, J. P. et al., *J. Immunol.* 147:1759 (1991)). Experiments further described in Example 3, in which methylation of bacterial DNA with CpG methylase was found to abolish mitogenicity, demonstrates that the difference in CpG status is the cause of B cell stimulation by bacterial DNA. This data supports the following conclusion: that unmethylated CpG dinucleotides present within bacterial DNA are responsible for the stimulatory effects of bacterial DNA.

Teleologically, it appears likely that lymphocyte activation by the CpG motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA, which would commonly be present in many anatomic regions and areas of inflammation due to apoptosis (cell death), would generally induce little or no lymphocyte activation due to CpG suppression and methylation. However, the presence of bacterial DNA containing unmethylated CpG motifs can cause lymphocyte activation precisely in infected anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. Since the CpG pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would receive one activation signal through cell membrane Ig and a second signal from bacterial DNA, and would therefore tend to be preferentially activated. The interrelationship of this pathway with other pathways of B cell activation provide a physiologic mechanism employing a polyclonal antigen to induce antigen-specific responses.

However, it is likely that B cell activation would not be totally nonspecific. B cells bearing antigen receptors specific for bacterial products could receive one activation signal through cell membrane Ig, and a second from bacterial DNA, thereby more vigorously triggering antigen specific immune responses. As with other immune defense mechanisms, the response to bacterial DNA could have undesirable consequences in some settings. For example, autoimmune responses to self antigens would also tend to be preferentially triggered by bacterial infections, since autoantigens could also provide a second activation signal to autoreactive B cells triggered by bacterial DNA. Indeed the induction of autoimmunity by bacterial infections is a common clinical observance. For example, the autoimmune disease systemic lupus erythematosus, which is: i) characterized by the production of anti-DNA antibodies; ii) induced by drugs which inhibit DNA methyltransferase (Cornacchia, E. J. et al., *J. Clin. Invest.* 92:38 (1993)); and iii) associated with reduced DNA methylation (Richardson, B., L. et al., *Arth. Rheum* 35:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by CpG motifs, as well as by binding of bacterial DNA to antigen receptors.

Further, sepsis, which is characterized by high morbidity and mortality due to massive and nonspecific activation of the immune system may be initiated by bacterial DNA and other products released from dying bacteria that reach concentrations sufficient to directly activate many lympho-

cytes. Further evidence of the role of CpG DNA in the sepsis syndrome is described in Cowdery, J., et. al., (1996) *The Journal of Immunology* 156:4570–4575.

Unlike antigens that trigger B cells through their surface Ig receptor, CpG-ODN did not induce any detectable Ca^{2+} flux, changes in protein tyrosine phosphorylation, or IP 3 generation. Flow cytometry with FITC-conjugated ODN with or without a CpG motif was performed sis described in Zhao, Q et al., (*Antisense Research and Development* 3:53–66 (1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for CpG ODN. Rather than acting through the cell membrane, that data suggests that unmethylated CpG containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. CpG ODN conjugated to either FITC or biotin retained full mitogenic properties, indicating no steric hindrance.

Recent data indicate the involvement of the transcription factor NFkB as a direct or indirect mediator of the CpG effect. For example, within 15 minutes of treating B cells or monocytes with CpG DNA, the level of NFkB binding activity is increased (FIG. 7). However, it is not increased by DNA that does not contain CpG motifs. In addition, it was found that two different inhibitors of NFkB activation, PDTC and gliotoxin, completely block the lymphocyte stimulation by CpG DNA as measured by B cell proliferation or monocytic cell cytokine secretion, suggesting that NFkB activation is required for both cell types.

There are several possible mechanisms through which NFkB can be activated. These include through activation of various protein kinases, or through the generation of reactive oxygen species. No evidence for protein kinase activation induced immediately after CpG DNA treatment of B cells or monocytic cells have been found, and inhibitors of protein kinase A, protein kinase C, and protein tyrosine kinases had no effects on the CpG induced activation. However, CpG DNA causes a rapid induction of the production of reactive oxygen species in both B cells and monocytic cells, as detected by the sensitive fluorescent dye dihydrorhodamine 123 as described in Royall, J. A., and Ischiropoulos, H. (*Archives of Biochemistry and Biophysics* 302:348–355 (1993)). Moreover, inhibitors of the generation of these reactive oxygen species completely block the induction of NFkB and the later induction of cell proliferation and cytokine secretion by CpG DNA.

Working backwards, the next question was how CpG DNA leads to the generation of reactive oxygen species so quickly. Previous studies by the inventors demonstrated that oligonucleotides and plasmid or bacterial DNA are taken up by cells into endosomes. These endosomes rapidly become acidified inside the cell. To determine whether this acidification step may be important in the mechanism through which CpG DNA activates reactive oxygen species, the acidification step was blocked with specific inhibitors of endosome acidification including chloroquine, monensin, and bafilomycin, which work through different mechanisms. FIG. 8A shows the results from a flow cytometry study using mouse B cells with the dihydrorhodamine 123 dye to determine levels of reactive oxygen species. The dye only sample in Panel A of the figure shows the background level of cells positive for the dye at 28.6%. As expected, this level of reactive oxygen species was greatly increased to 80% in the cells treated for 20 minutes with PMA and ionomycin, a positive control (Panel B). The cells treated with the CpG oligo also showed an increase in the level of reactive oxygen species such that more than 50% of the cells became positive (Panel D). However, cells treated with an oligonucleotide with the identical sequence except that the CpG was switched did not show this significant increase in the level of reactive oxygen species (Panel E).

In the presence of chloroquine, the results are very different (FIG. 8B). Chloroquine slightly lowers the background level of reactive oxygen species in the cells such that the untreated cells in Panel A have only 4.3% that are positive. Chloroquine completely abolishes the induction of reactive oxygen species in the cells treated with CpG DNA (Panel B) but does not reduce the level of reactive oxygen species in the cells treated with PMA and ionomycin (Panel E). This demonstrates that unlike the PMA plus ionomycin, the generation of reactive oxygen species following treatment of B cells with CpG DNA requires that the DNA undergo an acidification step in the endosomes. This is a completely novel mechanism of leukocyte activation. Chloroquine, monensin, and bafilomycin also appear to block the activation of NFkB by CpG DNA as well as the subsequent proliferation and induction of cytokine secretion.

Chronic Immune Activation by CpG DNA and Autoimmune Disorders

B cell activation by CpG DNA synergizes with signals through the B cell receptor. This raises the possibility that DNA-specific B cells may be activated by the concurrent binding of bacterial DNA to their antigen receptor, and by the co-stimulatory CpG-mediated signals. In addition, CpG DNA induces B cells to become resistant to apoptosis, a mechanism thought to be important for preventing immune responses to self antigens, such as DNA. Indeed, exposure to bDNA can trigger anti-DNA Ab production. Given this potential ability of CpG DNA to promote autoimmunity, it is therefore noteworthy that patients with the autoimmune disease systemic lupus erythematosus have persistently elevated levels of circulating plasma DNA which is enriched in hypomethylated CpGs. These findings suggest a possible role for chronic immune activation by CpG DNA in lupus etiopathogenesis.

A class of medications effective in the treatment of lupus is antimalarial drugs, such as chloroquine. While the therapeutic mechanism of these drugs has been unclear, they are known to inhibit endosomal acidification. Leukocyte activation by CpG DNA is not mediated through binding to a cell surface receptor, but requires cell uptake, which occurs via adsorptive endocytosis into an acidified chloroquine-sensitive intracellular compartment. This suggested the hypothesis that leukocyte activation by CpG DNA may occur in association with acidified endosomes, and might even be pH dependent. To test this hypothesis specific inhibitors of DNA acidicification were applied to determine whether B cells or monocytes could respond to CpG DNA if endosomal acidification was prevented.

The earliest leukocyte activation event that was detected in response to CpG DNA is the production of reactive oxygen species (ROS), which is induced within five minutes in primary spleen cells and both B and monocyte cell lines. Inhibitors of endosomal acidification including chloroquine, bafilomycin A, and monensin, which have different mechanisms of action, blocked the CpG-induced generation of ROS, but had no effect on ROS generation mediated by PMA, or ligation of CD40 or IgM. These studies show that ROS generation is a common event in leukocyte activation through diverse pathways. This ROS generation is generally

independent of endosomal acidification, which is required only for the ROS response to CpG DNA. ROS generation in response to CpG is not inhibited by the NFκB inhibitor gliotoxin, confirming that it is not secondary to NFκB activation.

To determine whether endosomal acidification of CpG DNA was also required for its other immune stimulatory effects were performed. Both LPS and CpG DNA induce similar rapid NFκB activation, increases in proto-oncogene mRNA levels, and cytokine secretion. Activation of NFκB by DNA depended on CpG motifs since it was not induced by bDNA treated with CpG methylase, nor by ODN in which bases were switched to disrupt the CpGs. Supershift experiments using specific antibodies indicated that the activated NFκB complexes included the p50 and p65 components. Not unexpectedly, NFκB activation in LPS- or CpG-treated cells was accompanied by the degradation of IκBα and IκBβ. However, inhibitors of endosomal acidification selectively blocked all of the CpG-induced but none of the LPS-induced cellular activation events. The very low concentration of chloroquine (<10 μM) that has been determined to inhibit CpG-mediated leukocyte activation is noteworthy since it is well below that required for antimalarial activity and oiler reported immune effects (e.g., 100–1000 μM). These experiments support the role of a pH-dependent signaling mechanism in mediating the stimulatory effects of CpG DNA.

drome and other diseases our studies suggest possible new therapeutic applications for antimalarial drugs that act as inhibitors of endosomal acidification.

CpG-induced ROS generation could be an incidental consequence of cell activation, or a signal that mediates this activation. The ROS scavenger N-acetyl-L-cysteine (NAC) blocks CpG-induced NFκB activation, cytokine production, and B cell proliferation, suggesting a causal role for ROS generation in these pathways. These data are compatible with previous evidence supporting a role for ROS in the activation of NfκB. WEHI-231 B cells (5×10^5 cells/ml) were precultured for 30 minutes with or without chloroquine (5 μg/ml [<10 μM]) or gliotoxin (0.2 μg/ml). Cell aliquots were then cultured as above for 10 minutes in RPMI medium with or without a CpG ODN (1826) or non-CpG ODN (1911) at 1 μM or phorbol myristate acetate (PMA) plus ionomycin (iono). Cells were then stained with dihydrorhodamine-123 and analyzed for intracellular ROS production by flow cytometry as described (A. K. Krieg, A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. Koretzky and D. Klinman, *Nature* 374, 546 (1995); Yi, A.-K., D. M. Klinman, T. L. Martin, S. Matson and A. M. Krieg, *J. Immunol.*, 157, 5394–5402 (1996); Krieg, A. M, *J. Lab. Clin. Med.*, 128, 128–133 (1996)). J774 cells, a monocytic line, showed similar pH-dependent CpG induced ROS responses. In contrast, CpG DNA did not induce the generation of extracellular ROS, nor any detect-

TABLE 15

Specific blockade of CpG-induced TNF-α and IL-12 expression by inhibitors of endosomal acidification or NFκB activation

	Medium		Inhibitors: Bafilomycin (250 nM)		Chloroquine (2.5 μg/ml)		Monensin (10 μM)		NAC (50 mM)	TPCK (50 μM)	Gliotoxin (0.1 μg/ml)	Bisgliotoxin (0.1 μg/ml)
activators	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	TNF-α	TNF-α	TNF-α
Medium	37	147	46	102	27	20	22	73	10	24	17	41
CpG ODN	455	17,114	71	116	28	6	49	777	54	23	31	441
LPS	901	22,485	1370	4051	1025	12418	491	4796	417	46	178	1120

TABLE 15 legend IL-12 and TNF-α assays: The murine monocyte cell line 3774 (1×10^5 cells/ml for IL 12 or 1×10^6 cells/ml for TNF-α), were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the CpG oligodeoxynucleotide (ODN) 1826 (TCCATGACGTTCCTGACGTT SEQ ID NO:10) at 2 μM or LPS (10 μg/ml) for 4 hr (TNF-α or 24 hr (IL-12) at which time the supernatant was harvested. ELISA for IL-12 or TNF-α (pg/ml) was performed on the supernatants essentially as described (A. K. Krieg, A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. Koretzky and D. Klinman, Nature 374, 546 (1995); Yi A.-K., D. M. Klinman, T. L. Martin, S. Matson and A. M. Krieg, J Immunol., 157, 5394–5402 (1996); Krieg, A. M, J Lab. Clin. Med., 128, 128–133 (1996). Cells cultured with ODN that lacked CpG motifs did not induce cytokine secretion. Similar specific inhibition of CpG responses was seen with IL-6 assays, and in experiments using primary spleen cells or the B cell lines CH12.LX and WEHI-231. 2.5 μg/ml of chloroquine is equivalent to <5 μM. Other inhibitors of NF-κB activation including PDTC and calpain inhibitors I and II gave similar results to the inhibitors shown. The results shown are representative of those obtained in ten different experiments.

Excessive immune activation by CpG motifs may contribute to the pathogenesis of the autoimmune disease systemic lupus erythematosus, which is associated with elevated levels of circulating hypomethylated CpG DNA. Chloroquine and related antimalarial compounds are effective therapeutic agents for the treatment of systemic lupus erythematosus and some other autoimmune diseases, although their mechanism of action has been obscure. Our demonstration of the ability of extremely low concentrations of chloroquine to specifically inhibit CpG-mediated leukocyte activation suggests a possible new mechanism for its beneficial effect. It is noteworthy that lupus recurrences frequently are thought to be triggered by microbial infection. Levels of bDNA present in infected tissues can be sufficient to induce a local inflammatory response. Together with the likely role of CpG DNA as a mediator of the sepsis syndrome and other diseases our studies suggest possible new therapeutic applications for antimalarial drugs that act as inhibitors of endosomal acidification.

CpG-induced ROS generation could be an incidental consequence of cell activation, or a signal that mediates this activation. The ROS scavenger N-acetyl-L-cysteine (NAC) blocks CpG-induced NFκB activation, cytokine production, and B cell proliferation, suggesting a causal role for ROS generation in these pathways. These data are compatible with previous evidence supporting a role for ROS in the activation of NfκB. WEHI-231 B cells (5×10^5 cells/ml) were precultured for 30 minutes with or without chloroquine (5 μg/ml [<10 μM]) or gliotoxin (0.2 μg/ml). Cell aliquots were then cultured as above for 10 minutes in RPMI medium with or without a CpG ODN (1826) or non-CpG ODN (1911) at 1 μM or phorbol myristate acetate (PMA) plus ionomycin (iono). Cells were then stained with dihydrorhodamine-123 and analyzed for intracellular ROS production by flow cytometry as described (A. K. Krieg, A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. Koretzky and D. Klinman, *Nature* 374, 546 (1995); Yi, A.-K., D. M. Klinman, T. L. Martin, S. Matson and A. M. Krieg, *J. Immunol.*, 157, 5394–5402 (1996); Krieg, A. M, *J. Lab. Clin. Med.*, 128, 128–133 (1996)). J774 cells, a monocytic line, showed similar pH-dependent CpG induced ROS responses. In contrast, CpG DNA did not induce the generation of extracellular ROS, nor any detectable neutrophil ROS. These concentrations of chloroquine (and those used with the other inhibitors of endosomal acidification) prevented acidification of the internalized CpG DNA using fluorescein conjugated ODN as described by Tonkinson, et al., (*Nucl. Acids Res.* 22, 4268 (1994); A. M. Krieg, In: *Delivery Strategies for Antisense Oligonucleotide Therapeutics.* Editor, S. Akhtar, CRC Press, Inc., pp. 177 (1995)). At higher concentrations than those required to inhibit endosomal acidification, nonspecific inhibitory effects were observed. Each experiment was performed at least three times with similar results.

While NFκB is known to be an important regulator of gene expression, it's role in the transcriptional response to CpG DNA was uncertain. To determine whether this NFκB activation was required for the CpG mediated induction of

gene expression cells were activated with CpG DNA in the presence or absence of pyrrolidine dithiocarbamate (PDTC), an inhibitor of IκB phosphorylation. These inhibitors of NFκB activation completely blocked the CpG-induced expression of protooncogene and cytokine mRNA and protein, demonstrating the essential role of NFκB as a mediator of these events. None of the inhibitors reduced cell viability under the experimental conditions used in these studies. A J774, a murine monocyte cell line, was cultured in the presence of calf thymus (CT), *E. coli* (EC), or methylated *E. coli* (mEC) DNA (methylated with CpG methylase as described[4]) at 5 μg/ml or a CpG oligodeoxynucleotide (ODN 1826; Table 15) or a non-CpG ODN (ODN 1745; TCCATGAGCTTCCTGAGTCT; SEQ ID NO:8) at 0.75 μM for 1 hr, following which the cells were lysed and nuclear extracts prepared. A doublestranded ODN containing a consensus NFκB site was 5' radiolabeled and used as a probe for EMSA essentially as described (J. D. Dignam, R. M. Lebovitz and R. G. Roeder, *Nucleic Acids Res.* 11, 1475 (1983); M. Briskin, M. Damore, R. Law, G. Lee, P. W. Kincade, C. H. Sibley, M. Kuehl and R. Wall, *Mol. Cell. Biol.* 10, 422 (1990)). The position of the p50/p65 heterodimer was determined by supershifting with specific Ab to p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, Calif.). Chloroquine inhibition of CpG-induced but not LPS-induced NFκB activation was established using J774 cells. The cells were precultured for 2 hr in the presence or absence of chloroquine (20 μg/ml) and then stimulated as above for 1 hr with either EC DNA, CpG ODN, non-CpG ODN or LPS (1 μg/ml). Similar chloroquine sensitive CpG-induced activation of NFκB was seen in a B cell line, WEHI-231 and primary spleen cells. These experiments were performed three times over a range of chloroquine concentrations from 2.5 to 20 μg/ml with similar results.

It was also established that CpG-stimulated mRNA expression requires endosomal acidification and NFκB activation in B cells and monocytes. J774 cells (2×10^6 cells/ml) were cultured for 2 hr in the presence or absence of chloroquine (2.5 μg/ml [<5 μM]) or N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; 50 μM), a serine/threonine protease inhibitor that prevents IκB proteolysis and thus blocks NFκB activation. Cells were then stimulated with the addition of *E. coli* DNA (EC; 50 μg/ml), calf thymus DNA (CT; 50 μg/ml), LPS (10 μg/ml), CpG ODN (1826; 1 μM), or control non-CpG ODN (1911; 1 μM) for 3 hr. WEHI-231 B cells (5×10^5 cells/ml) were cultured in the presence or absence of gliotoxin (0.1 μg/ml) or bisgliotoxin (0.1 μg/ml) for 2 hrs and then stimulated with a CpG ODN (1826), or control non-CpG ODN (1911; TCCAGGACTTTCCTCAGGTT; SEQ ID NO:107) at 0.5 μM for 8 hr. In both cases, cells were harvested and RNA was prepared using RNAzol following the manufacturer's protocol. Multi-probe RNase protection assay was performed as described (A.-K. Yi, P. Hornbeck, D. E. Lafrenz and A. M. Krieg, *J. Immunol.*, 157, 4918–4925 (1996). Comparable amounts of RNA were loaded into each lane by using ribosomal μRNA as a loading control (L32). These experiments were performed three tides with similar results.

The results indicate that leukocytes respond to CpG DNA through a novel pathway involving the pH-dependent generation of intracellular ROS. The pH dependent step may be the transport or processing of the CpG DNA, the ROS generation, or some other event. ROS are widely thought to be second messengers in signaling pathways in diverse cell types, but have not previously been shown to mediate a stimulatory signal in B cells.

Presumably, there is a protein in or near the endosomes that specifically recognizes DNA containing CpG motifs and leads to the generation of reactive oxygen species. To detect any protein in the cell cytoplasm that may specifically bind CpG DNA, electrophoretic mobility shift assays (EMSA) were used with 5' radioactively labeled oligonucleotides with or without CpG motifs. A band was found that appears to represent a protein binding specifically to single stranded oligonucleotides that have CpG motifs, but not to oligonucleotides that lack CpG motifs or to oligonucleotides in which the CpG motif has been methylated. This binding activity is blocked if excess of oligonucleotides that contain the NFkB binding site was added. This suggests that an NFkB or related protein is a component of a protein or protein complex that binds the stimulatory CpG oligonucleotides.

No activation of CREB/ATF proteins was found at time points where NFkB was strongly activated. These data therefore do not provide proof that NFkB proteins actually bind to the CpG nucleic acids, but rather that the proteins are required in some way for the CpG activity. It is possible that a CREB/ATF or related protein may interact in some way with NFkB proteins or other proteins thus explaining the remarkable similarity in the binding motifs for CREB proteins and the optimal CpG motif. It remains possible that the oligos bind to a CREB/ATF or related protein, and that this leads to NFkB activation.

Alternatively, it is very possible that the CpG nucleic acids may bind to one of the TRAF proteins that bind to the cytoplasmic region of CD40 and mediate NFkB activation when CD40 is cross-linked. Examples of such TRAF proteins include TRAF-2 and TRAF-5.

Method for Making Immunostimulatory Nucleic Acids

For use in the instant invention, nucleic acids can be synthesized de novo using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (S. L. Beaucage and M. H. Caruthers, (1981) *Tet. Let.* 22:1859); nucleoside H-phosphonate method (Garegg et al., (1986) *Tet. Let.* 27: 4051–4054; Froehler et al., (1986) *Nucl. Acid. Res.* 14: 5399–5407; Garegg et al., (1986) *Tet. Let.* 27: 4055–4058, Gaffney et al., (1988) *Tet. Let.* 29:2619–2622). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, oligonucleotides can be prepared from existing nucleic acid sequences (e.g. genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

For use in vivo, nucleic acids are preferably relatively resistant to degradation (e.g. via endo- and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid has at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made e.g. as described in U.S. Pat. No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A. (1990) *Chem. Rev.* 90:544; Goodchild, J. (1990) *Bioconju-*

gate Chem. 1:165). 2'-O-methyl nucleic acids with CpG motifs also cause immune activation, as do ethoxy-modified CpG nucleic acids. In fact, no backbone modifications have been found that completely abolish the CpG effect, although it is greatly reduced by replacing the C with a 5-methyl C.

For administration in vivo, nucleic acids may be associated with a molecule that results in higher affinity binding to target cell (e.g. B-cell, monocytic cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells to form a "nucleic acid delivery complex". Nucleic acids can be ionically, or covalently associated with appropriate molecules using techniques which are well known in the art. A variety of coupling or crosslinking agents can be used e.g. protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Nucleic acids can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

Therapeutic Uses of Immunostimulatory Nucleic Acid Molecules

Based on their immunostimulatory properties, nucleic acid molecules containing at least one unmethylated CpG dinucleotide can be administered to a subject in vivo to treat an "immune system deficiency". Alternatively, nucleic acid molecules containing at least one unmethylated CpG dinucleotide can be contacted with lymphocytes (e.g. B cells, monocytic cells or NK cells) obtained from a subject having an immune system deficiency ex vivo and activated lymphocytes can then be re-implanted in the subject.

As reported herein, in response to unmethylated CpG containing nucleic acid molecules, an increased number of spleen cells secrete IL-6, IL-12, IFN-γ, IFN-α, IFN-β, IL-1, IL-3, IL-10, TNF-α, TNF-β, GM-CSF, RANTES, and probably others. The increased IL-6 expression was found to occur in B cells, $CD4^+$ T cells and monocytic cells.

Immunostimulatory nucleic acid molecules can also be administered to a subject in conjunction with a vaccine to boost a subject's immune system and thereby effect a better response from the vaccine. Preferably the immunostimulatory nucleic acid molecule is administered slightly before or at the same time as the vaccine. A conventional adjuvant may optionally be administered in conjunction with the vaccine, which is minimally comprised of an antigen, as the conventional adjuvant may further improve the vaccination by enhancing antigen absorption.

When the vaccine is a DNA vaccine at least two components determine its efficacy. First, the antigen encoded by the vaccine determines the specificity of the immune response. Second, if the backbone of the plasmid contains CpG motifs, it functions as an adjuvant for the vaccine. Thus, CpG DNA acts as an effective "danger signal" and causes the immune system to respond vigorously to new antigens in the area. This mode of action presumably results primarily from the stimulatory local effects of CpG DNA on dendritic cells and other "professional" antigen presenting cells, as well as from the co-stimulatory effects on B cells.

Immunostimulatory oligonucleotides and unmethylated CpG containing vaccines, which directly activate lymphocytes and co-stimulate an antigen-specific response, are fundamentally different from conventional adjuvants (e.g. aluminum precipitates), which are inert when injected alone and are thought to work through absorbing the antigen and thereby presenting it more effectively to immune cells. Further, conventional adjuvants only work for certain antigens, only induce an antibody (humoral) immune response (Th2), and are very poor at inducing cellular immune responses (Th1). For many pathogens, the humoral response contributes little to protection, and can even be detrimental.

In addition, an immunostimulatory oligonucleotide can be administered prior to, along with or after administration of a chemotherapy or immunotherapy to increase the responsiveness of the malignant cells to subsequent chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. CpG nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and ADCC may likewise be beneficial in cancer immunotherapy, alone or in conjunction with other treatments.

Another use of the described immunostimulatory nucleic acid molecules is in desensitization therapy for allergies, which are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated CpG nucleic acids, are predominantly of a class called "Th1" which is most marked by a cellular immune response and is associated with IL-12 and IFN-γ. The other major type of immune response is termed a Th2 immune response, which is associated with more of an antibody immune response and with the production of IL4, IL-5 and IL-10. In general, it appears that allergic diseases are mediated by Th2 type immune responses and autoimmune diseases by Th1 immune response. Based on the ability of the immunostimulatory nucleic acid molecules to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of an immunostimulatory nucleic acid (or a vector containing a nucleic acid) alone or in conjunction with an allergen can be administered to a subject to treat or prevent an allergy.

Nucleic acids containing unmethylated CpG motifs may also have significant therapeutic utility in the treatment of asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotype switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN-γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines.

As described in detail in the following Example 12, oligonucleotides containing an unethylated CpG motif (i.e., TCCATGACGTTCCTGACGTT; SEQ D NO. 10), but not a control oligonucleotide (TCCATGAGCTTCCTGAGTCT; SEQ ID NO 8) prevented the development of an inflammatory cellular infiltrate and eosinophilia in a murine model of asthma. Furthermore, the suppression of eosinophilic inflammation was associated with a suppression of a Th2 response and induction of a Th1 response.

For use in therapy, an effective amount of an appropriate immunostimulatory nucleic acid molecule alone or formulated as a delivery complex can be administered to a subject by any mode allowing the oligonucleotide to be taken up by the appropriate target cells (e.g. B-cells and monocytic cells). Preferred routes of administration include oral and transdermal (e.g. via a patch). Examples of other routes of administration include injection (subcutaneous, intravenous, pareniteral, intraperitoneal, intrathecal, etc.). The injection can be in a bolus or a continuous infusion.

A nucleic acid alone or as a nucleic acid delivery complex can be administered in conjunction with a pharmaceutically acceptable carrier. As used herein, the phrase "pharmaceu-

tically acceptable carrier" is intended to include substances that can be coadministered with a nucleic acid or a nucleic acid delivery complex and allows the nucleic acid to perform its indicated function. Examples of such carriers include solutions, solvents, dispersion media, delay agents, emulsions and the like. The use of such media for pharmaceutically active substances are well known in the art. Any other conventional carrier suitable for use with the nucleic acids falls within the scope of the instant invention.

The term "effective amount" of a nucleic acid molecule refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a nucleic acid containing at least one unmethylated CpG for treating an immune system deficiency could be that amount necessary to eliminate a tumor, cancer, or bacterial, viral or fungal infection. An effective amount for use as a vaccine adjuvant could be that amount useful for boosting a subjects immune response to a vaccine. An "effective amount" for treating asthma can be that amount useful for redirecting a Th2 type of immune response that is associated with asthma to a Th1 type of response. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular nucleic acid being administered (e.g. the number of unmethylated CpG motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular oligonucleotide without necessitating undue experimentation.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example 1

Effects of ODNs on B Cell Total RNA Synthesis and Cell Cycle

B cells were purified from spleens obtained from 6–12 wk old specific pathogen free DBA/2 or BXSB mice (bred in the University of Iowa animal care facility; no substantial strain differences were noted) that were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over lymphocyte M (Cedarlane Laboratories, Homby, Ontario, Canada) ("B cells"). B cells contained fewer than 1% $CD4^+$ or $CD8^+$ cells. 8×10^4 B cells were dispensed in triplicate into 96 well microtiter plates in 100 μl RPMI containing 10% FBS (heat inactivated to 65° C. for 30 min.), 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamate. 20 μM ODN were added at the start of culture for 20 h at 37° C., cells pulsed with 1 μCi of ^3H uridine, and harvested and counted 4 hr later. Ig secreting B cells were enumerated using the ELISA spot assay after culture of whole spleen cells with ODN at 20 μM for 48 hr. Data, reported in Table 1, represent the stimulation index compared to cells cultured without ODN. ^3H thymidine incorporation assays showed similar results, but with some nonspecific inhibition by thymidine released from degraded ODN (Matson. S and A. M. Krieg (1992) Nonspecific suppression of ^3H-thymidine incorporation by control oligonucleotides. *Antisense Research and Development* 2:325).

Example 2

Effects of ODN on Production of IgM from B Cells

Single cell suspensions from the spleens of freshly killed mice were treated with anti-Thyl, anti-CD4, and anti-CD8 and complement by the method of Leibson et al., *J. Exp. Med.* 154:1681 (1981)). Resting B cells (<02% T cell contamination) were isolated from the 63–70% band of a discontinuous Percoll gradient by the procedure of DeFranco et al, *J. Exp. Med.* 155:1523 (1982). These were cultured as described above in 30 μM ODN or 20 μg/ml LPS for 48 hr. The number of B cells actively secreting IgM was maximal at this time point, as determined by ELIspot assay (Klinman, D. M. et al. *J. Immunol.* 144:506 (1990)). In that assay, B cells were incubated for 6 hrs on anti-Ig coated microtiter plates. The Ig they produced (>99% IgM) was detected using phosphatase-labeled anti-Ig (Southern Biotechnology Associated, Birmingham, Ala.). The antibodies produced by individual B cells were visualized by addition of BCIP (Sigma Chemical Co., St. Louis Mo.) which forms an insoluble blue precipitate in the presence of phosphatase. The dilution of cells producing 20–40 spots/well was used to determine the total number of antibody-secreting B cells/sample. All assays were performed in triplicate (data reported in Table 1). In some experiments, culture supernatants were assayed for IgM by ELISA, and showed similar increases in response to CpG-ODN.

Example 3

B Cell Stimulation by Bacterial DNA

DBA/2 B cells were cultured with no DNA or 50 μg/ml of a) *Micrococcus lysodeikticus*; b) NZB/N mouse spleen; and c) NFS/N mouse spleen genomic DNAs for 48 hours, then pulsed with ^3H thymidine for 4 hours prior to cell harvest. Duplicate DNA samples were digested with DNASE I for 30 minutes at 37 C prior to addition to cell cultures. *E. coli* DNA al5so induced an 8.8 fold increase in the number of IgM secreting B cells by 48 hours using the ELISA-spot assay.

DBA/2 B cells were cultured with either no additive, 50 μg/ml LPS or the ODN 1; 1a; 4; or 4a at 20 uM. Cells were cultured and harvested at 4, 8, 24 and 48 hours. BXSB cells were cultured as in Example 1 with 5, 10, 20, 40 or 80 μM of ODN 1; la; 4; or 4a or LPS. In this experiment, wells with no ODN had 3833 cpm. Each experiment was performed at least three times with similar results. Standard deviations of the triplicate wells were <5%.

Example 4

Effects of ODN on Natural Killer (NK) activity

10×10^6 C57BL/6 spleen cells were cultured in two ml RPMI (supplemented as described for Example 1) with or without 40 μM CpG or non-CpG ODN for forty-eight hours. Cells were washed, and then used as effector cells in a short term ^{51}Cr release assay with YAC-1 and 2C11, two NK sensitive target cell lines (Ballas, Z. K. et al. (1993) *J. Immunol.* 150:17). Effector cells were added at various concentrations to 10^4 ^{51}Cr-labeled target cells in V-bottom microtiter plates in 0.2 ml, and incubated in 5% CO_2 for 4 hr. at 37° C. Plates were then centrifuged, and an aliquot of the supernatant counted for radioactivity. Percent specific lysis was determined by calculating the ratio of the ^{51}Cr released in the presence of effector cells minus the ^{51}Cr

released when the target cells are cultured alone, over the total counts released after cell lysis in 2% acetic acid minus the ^{51}Cr cpm released when the cells are cultured alone.

Example 5

In vivo Studies with CpG Phosphorothioate ODN

Mice were weighed and injected IP with 0.25 ml of sterile PBS or the indicated phophorothioate ODN dissolved in PBS. Twenty four hours later, spleen cells were harvested, washed, and stained for flow cytometry using phycoerythrin conjugated 6B2 to gate on B cells in conjunction with biotin conjugated anti Ly-6A/E or anti-Ia^d (Pharmingen, San Diego, Calif.) or anti-Bla-1 (Hardy, R. R. et al., *J. Exp. Med.* 159:1169 (1984). Two mice were studied for each condition and analyzed individually.

Example 6

Titration of Phosphorothioate ODN for B Cell Stimulation

B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the CpG ODN 1d and 3Db and then either pulsed after 20 hr with ^3H uridine or after 44 hr with ^3H thymidine before harvesting and determining cpm.

Example 7

Rescue of B Cells From Apoptosis

WEHI-231 cells (5×10^4/well) were cultured for 1 hr. at 37 C. in the presence or absence of LPS or the control ODN 1a or the CpG ODN 1d and 3Db before addition of anti-IgM (1 μ/ml). Cells were cultured for a further 20 hr. before a 4 hr. pulse with 2 μCi/well ^3H thymidine. In this experiment, cells with no ODN or anti-IgM gave 90.4×10^3 cpm of ^3H thymidine incorporation by addition of anti-IgM. The phosphodiester ODN shown in Table 1 gave similar protection, though with some nonspecific suppression due to ODN degradation. Each experiment was repeated at least 3 times with similar results.

Example 8

In vivo Induction of Murine IL-6

DBA/2 female mice (2 mos. old) were injected IP with 500 g CpG or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each time point. IL-6 was measured by Elisa, and IL-6 concentration was calculated by comparison to a standard curve generated using recombinant IL-6. The sensitivity of the assay was 10 pg/ml. Levels were undetectable after 8 hr.

Example 9

Systemic Induction of Murine IL-6 Transcription

Mice and cell lines. DBA/2, BALB/c, and C3H/HeJ mice at 5–10 wk of age were used as a source of lymphocytes. All mice were obtained from The Jackson Laboratory (Bar Harbor, Me.), and bred and maintained under specific pathogen-free conditions in the University of Iowa Animal Care Unit. The mouse B cell line CH12.LX was kindly provided by Dr. G. Bishop (University of Iowa, Iowa City).

Cell preparation. Mice were killed by cervical dislocation. Single cell suspensions were prepared aseptically from the spleens from mice. T cell depleted mouse splenocytes were prepared by using anti-Thy-1.2 and complement and centrifugation over lymphocyte M (Cedarlane Laboratories, Homby, Ontario, Canada) as described (Krieg, A. M. et al., (1989) A role for endogenous retroviral sequences in the regulation of lymphocyte activation. *J. Immunol.* 143:2448).

ODN and DNA. Phosphodiester oligonucleotides (O-ODN) and the backbone modified phosphorothioate oligonucleotides (S-ODN) were obtained from the DNA Core facility at the University of Iowa or from Operon Technologies (Alameda, Calif.). *E. coli* DNA (Strain B) and calf thymus DNA were purchased from Sigma (St. Louis, Mo.). All DNA and ODN were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and/or ethanol precipitation. *E. coli* and calf thymus DNA were single stranded prior to use by boiling for 10 min. followed by cooling on ice for 5 min. For some experiments, *E. coli* and calf thymus DNA were digested with DNase I (2U/μg of DNA) at 37° C. for 2 hr in 1×SSC with 5 mM MgCl2. To methylate the cytosine in CpG dinucleotides in *E. coli* DNA, *E. coli* DNA was treated with CpG methylase (M. SssI; 2 U/μg of DNA) in NEBuffer 2 supplemented with 160 μM S-adenosyl methionine and incubated overnight at 37° C. Methylated DNA was purified as above. Efficiency of methylation was confirmed by Hpa II digestion followed by analysis by gel electrophoresis. All enzymes were purchased from New England Biolabs (Beverly, Mass.). LPS level in ODN was less than 12.5 ng/mg and *E. coli* and calf thymus DNA contained less than 2.5 ng of LPS/mg of DNA by Limulus assay.

Cell Culture. All cells were cultured at 37° C. in a 5% CO_2 humidified incubator maintained in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 50 μg/ml), CpG or non-CpG phosphodiester ODN (O-ODN) (20 μM), phosphorothioate ODN (S-ODN) (0.5 μM), or *E. coli* or calf thymus DNA (50 μg/ml) at 37° C. for 24 hr. (for IL-6 production) or 5 days (for IgM production). Concentrations of stimulants were chosen based on preliminary studies with titrations. In some cases, cells were treated with CpG O-ODN along with various concentrations (1–10 μg/ml) of neutralizing rat IgG1 antibody against murine IL-6 (hybridoma MP5-20F3) or control rat IgG1 mAb to *E. coli* b-galactosidase (hybridoma GL113; ATCC, Rockville, Md.) (20) for 5 days. At the end of incubation, culture supernatant fractions were analyzed by ELISA as below.

In vivo induction of IL-6 and IgM. BALB/c mice were injected intravenously (iv) with PBS, calf thymus DNA (200 μg/100 μl PBS/mouse), *E. coli* DNA (200 μg/100 μl PBS/mouse), or CpG or non-CpG S-ODN (200 μg/100 μl PBS/mouse). Mice (two/group) were bled by retroorbital puncture and sacrificed by cervical dislocation at various time points. Liver, spleen, thymus, and bone marrow were removed and RNA was prepared from those organs using RNAzol B (Tel-Test, Friendswood, Tex.) according to the manufacturers protocol.

ELISA. Flat-bottomed Immun 1 plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 100 μl/well of anti-mouse IL-6 mAb (MP5-20F3) (2 μg/ml) or anti-mouse IgM μ-chain specific (5 μg/ml; Sigma, St. Louis, Mo.) in carbonate-bicarbonate, pH 9.6 buffer (15 nM Na_2CO_3, 35 mM $NaHC_3$) overnight at 4° C. The plates were then washed with TPBS (0.5 mM $MgCl_2o6H_2O$, 2.68 mM KCl, 1.47 mM KH_2PO_4, 0.14 M NaCl, 6.6 mM , K_2HPO_4, 0.5% Tween 20) and blocked with 10% FCS in TPBS for 2 hr at room temperature and then washed again. Culture supernatants, mouse sera, recombinant mouse IL-6

(Pharmingen, San Diego, Calif.) or purified mouse IgM (Calbiochem, San Diego, Calif.) were appropriately diluted in 10% FCS and incubated in triplicate wells for 6 hr at room temperature. The plates were washed and 100 μl/well of biotinylated rat anti-mouse IL-6 monoclonal antibodies (MP5-32C11, Pharmingen, San Diego, Calif.) (1 μg/ml in 10% FCS) or biotinylated anti-mouse Ig (Sigma, St. Louis, Mo.) were added and incubated for 45 min. at room temperature following washes with TPBS. Horseradish peroxidase (HRP) conjugated avidin (Bio-rad Laboratories, Hercules, Calif.) at 1:4000 dilution in 10% FCS (100 μl/well) was added and incubated at room temperature for 30 min. The plates were washed and developed with o-phenylendiamine dihydrochloride (OPD; Sigma, St. Louis, Mo.) 0.05 M phosphate-citrate buffer, pH 5.0, for 30 min. The reaction was stopped with 0.67 N H_2SO_4 and plates were read on a microplate reader (Cambridge Technology, Inc., Watertown, Mass.) at 490–600 nm. The results are shown in FIGS. 1 and 2.

RT-PCR. A sense primer, an antisense primer, and an internal oligonucleotide probe for IL-6 were synthesized using published sequences (Montgomery, R. A. and M. S. Dallman (1991), Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction (*J. Immunol.)* 147:554). cDNA synthesis and IL-6 PCR was done essentially as described by Montgomery and Dalhman (Montgomery, R. A. and M. S. Dalhman (1991), Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction (*J. Immunol.)* 147:554) using RT-PCR reagents from Perkin-Elmer Corp. (Hayward, Calif.). Samples were analyzed after 30 cycles of amplification by gel electrophoresis followed by unblot analysis (Stoye, J. P. et al., (1991) DNA hybridization in dried gels with fragmented probes: an improvement over blotting techniques, *Techniques* 3:123). Briefly, the gel was hybridized at room temperature for 30 min. in denaturation buffer (0.05 M NaOH, 1.5M NaCl) followed by incubation for 30 min. in renaturation buffer (1.5 M NaCl, 1 M Tris, pH 8) and a 30 min. wash in double distilled water. The gel was (tried and prehybridized at 47° C. for 2 hr. hybridization buffer (5×SSPE, 0.1% SDS) containing 10 μg/ml denatured salmon sperm DNA. The gel was hybridized with 2×10^6 cpm/ml g-[^{32} P]ATP end-labeled internal oligonucleotide probe for IL-6 (5'CATTTCCACGATTTCCCA3') SEQ ID. No. 109) overnight at 47° C., washed 4 times (2×SSC, 0.2% SDS) at room temperature and autoradiographed. The results are shown in FIG. 3.

Cell Proliferation assay. DBA/2 mice spleen B cells (5×10^4 cells/100 μl/well) were treated with media, CpG or non-CpG S-ODN (0.5 μM) or O-ODN (20 μM) for 24 hr at 37° C. Cells were pulsed for the last four hr. with either [^{3}H] Thymidine or [^{3}H] Uridine (1 μCi/well). Amounts of [^{3}H] incorporated were measured using Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, Ill.).

Transfections and CAT assays. WEHI-231 cells (10^7cells) were electroprated with 20 μg of control or human IL-6 promoter-CAT construct (kindly provided by S. Manolagas, Univ. of Arkansas) (Pottratz, S. T. et al., (1994) 17B-estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. *J. Clin. Invest.* 93:944) at 250 mV and 960 μF. Cells were stimulated with various concentrations or CpG or non-CpG ODN after electroporation. Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (Seed, B. and J. Y. Sheen (1988) A single phase-extraction assay for chloramphenicol acetyl transferase activity. *Gene* 76:271) 16 hr. after transfection. The results are presented in FIG. 5.

Example 10

Oligodeoxynucleotide Modifications Determine the Magnitude of B Cell Stimulation by CpG Motifs

ODN were synthesized on an Applied Biosystems Inc. (Foster City, Calif.) model 380A, 380B, or 394 DNA synthesizer using standard procedures (Beacage and Caruthers (1981) Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Letters 22, 1859–1862.). Phosphodiester ODN were synthesized using standard beta-cyanoethyl phosphoramidite chemistry. Phosphorothioate linkages were introduced by oxidizing the phosphite linkage with elemental sulfur instead of the standard iodine oxidation. The four common nucleoside phosphoramidites were purchased from Applied Biosystems. All phosphodiester and thioate containing ODN were deprotected by treatment with concentrated ammonia at 55° C. for 12 hours. The ODN were purified by gel exclusion chromatography and lyophilized to dryness prior to use. Phosphorodithioate linkages were introduced by using deoxynucleoside S-(b-benzoyhnercaptoethyl) pyrrolidino thiophosphoramidites (Wiesler, W. T. et al., (1993) In Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs—Synthesis and Properties, Agrawal, S. (ed.), Humana Press, 191–206.). Dithioate containing ODN were deprotected by treatment with concentrated ammonia at 55° C. for 12 hours followed by reverse phase HPLC purification.

In order to synthesize oligomers containing methylphosphonothioates or methylphosphonates as well as phosphodiesters at any desired internucleotide linkage, two different synthetic cycles were used. The major synthetic differences in the two cycles are the coupling reagent where dialkylaminomethylnucleoside phosphines are used and the oxidation reagents in the case of methylphosphonothioates. In order to synthesize either derivative, the condensation time has been increased for the dialkylaminomethylnucleoside phosphines due to the slower kinetics, of coupling (Jager and Engels, (1984) Synthesis of deoxynucleoside methylphosphonates via a phosphonamidite approach. Tetrahedron Letters 24, 1437–1440). After the coupling step has been completed, the methylphosphinodiester is treated with the sulfurizing reagent (5% elemental sulfur, 100 millimolar N,N-diamethylaminopyridine in carbon disulfide/pyridine/triethylamine), four consecutive times for 450 seconds each to produce methylphosphonothioates. To produce methylphosphonate linkages, the methylphosphinodiester is treated with standard oxidizing reagent (0.1 M iodine in tetrahydrofuran/2,6-lutidine/water).

The silica gel bound oligomer was treated with distilled pyridine/concentrated ammonia, 1:1, (v/v) for four days at 4 degrees centigrade. The supernatant was dried in vacuo, dissolved in water and chromatographed on a G50/50 Sephadex column.

As used herein, O-ODN refers to ODN which are phosphodiester; S-ODN are completely phosphorothioate modified; S-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5' and five 3' linkages are phosphorothioate modified; S_2-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5' and five 3' linkages are phosphorodithioate modified; and MP-O-ODN are chimeric ODN in which the central linkages are phosphodiester, but the two 5' and five 3' linkages are methylphosphonate modified. The ODN sequences studied (with CpG dinucleotides indicated by underlining) include:

3D (5" GAGAACCTGGACCTTCCAT), (SEQ. ID. NO. 20);

3M (5' TCCATGTCGTCCTGATGCT), (SEQ. ID. NO. 28);

5 (5' GGCGTTATTCCTGACTCGCC), (SEQ. ID. NO. 110); and

6 (5' CCTACGTTGTATGCGCCCAGCT), (SEQ. ID. NO. 111).

These sequences are representative of literally hundreds of CpG and non-CpG ODN that have been tested in the course of these studies.

Mice. DBA/2, or BXSB mice obtained from The Jackson Laboratory (Bar Harbor, Me.), and maintained under specific pathogen-free conditions were used as a source of lymphocytes at 5–10 wk of age with essentially identical results.

Cell proliferation assay. For cell proliferation assays, mouse spleen cells (5×10^4 cells/100 μl/well) were cultured at 37° C. in a 5% CO_2 humidified incubator in RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (heated to 65° C. for experiments with O-ODN, or 56° C. for experiments using only modified ODN), 1.5 μM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin for 24 hr or 48 hr as indicated. 1 μCi of ^3H uridine or thymidine (as indicated) was added to each well, and the cells harvested after an additional 4 hours of culture. Filters were counted by scintillation counting. Standard deviations of the triplicate wells were <5%. The results are presented in FIGS. 6–8.

Example 11

Induction of NK Activity

Phosphodiester ODN were purchased from Operon Technologies (Alameda, Calif.). Phosphorothioate ODN were purchased from the DNA core facility, University of Iowa, or from The Midland Certified Reagent Company (Midland. Tex.). *E.coli* (strain B) DNA and calf thymus DNA were purchased from Sigma (St. Louis, Mo.). All DNA and ODN were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and/or ethanol precipitation. The LPS level in ODN was less than 12.5 ng/mg and *E.coli* and calf thymus DNA contained less than 2.5 ng of LPS/mg of DNA by Limulus assay.

Virus-free, 4–6 week old, DBA/2, C57BL/6 (B6) and congenitally athymic BALB/C mice were obtained on contract through the Veterans Affairs from the National Cancer Institute (Bethesda, Md.). C57BL/6 SCID mice were bred in the SPF barrier facility at the University of Iowa Animal Care Unit.

Human peripheral mononuclear blood leukocytes PBMC) were obtained as previously described (Ballas, Z. K. et al., (1990) *J. Allergy Clin. Immunol.* 85:453; Ballas, Z. K. and W. Rasmussen (1990) *J. Immunol.* 145:1039; Ballas, Z. K. and W. Rasmussen (1993) *J. Immunol.* 150;17). Human or murine cells were cultured at 5×10^6/well, at 37° C. in a 5% CO_2 humidified atmosphere in 24-well plates (Ballas, Z. K. et al., (1990) *J. Allergy Clin. Immunol.* 85:453; Ballas, Z. K. and W. Rasmussen (1990) *J. Immunol* 145:1039; and Ballas, Z. K. and W. Rasmussen (1993) *J. Immunol*, 150:17), with medium alone or with CpG or non-CpG ODN at the indicated concentrations, or with *E.coli* or calf thymus (50 μg/ml) at 37° C. for 24 hr. All cultures were harvested at 18 hr. and the cells were used as effectors in a standard 4 hr. ^5Cr-release assay against K562 (human) or YAC-1 (mouse) target cells as previously described. For calculation of lytic units (LU), 1 LU was defined as the number of cells needed to effect 30% specific lysis. Where indicated, neutralizing antibodies against IFN-β (Lee Biomolecular, San Diego, Calif.) or IL-12 (C15.1, C15.6, C17.8, and C17.15; provided by Dr. Giorgio Trinchieri, The Wistar Institute, Philadelphia, Pa.) or their isotype controls were added at the initiation of cultures to a concentration of 10 μg/ml. For anti-IL-12 addition, 10 μg of each of the 4 MAB (or isotype controls) were added simultaneously. Recombinant human IL-2 was used at a concentration of 100 U/ml.

Example 12

Prevention of the Development of an Inflammatory Cellular Infiltrate and Eosinophilia in a Murine Model of Asthma

6–8 week old C56BL/6 mice (from The Jackson Laboratory, Bar Harbor, Me.) were immunized with 5,000 *Schistosoma mansoni* eggs by intraperitoneal (i.p.) injection on days 0 and 7. *Schistosoma mansoni* eggs contain an antigen (*Schistosoma mansoni* egg antigen (SEA)) that induces a Th2 immune response (e.g. production of IgE antibody). IgE antibody production is known to be an important cause of asthma.

The immunized mice were then treated with oligonucleotides (30 μg in 200 μl saline by i.p.injection), which either contained an unmethylated CpG motif (i.e., TCCATGACGTTCCTGACGTT; SEQ ID NO.10) or did not (i.e., control, TCCATGAGCTTCCTGAGTCT; SEQ ID NO.8). Soluble SEA (10 μg in 25 μl of saline) was administered by intranasal instillation on days 14 and 21. Saline was used as a control.

Mice were sacrificed at various times after airway challenge. Whole lung lavage was performed to harvest airway and alveolar inflammatory cells. Cytokine levels were measured from lavage fluid by ELISA. RNA was isolated from whole lung for Northern analysis and RT-PCR studies using CsCl gradients. Lungs were inflated and perfused with 4% paraformaldehyde for histologic examination.

FIG. 9 shows that when the mice are initially injected with the eggs i.p., and then inhale the egg antigen (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated CpG motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg antigen (open triangles).

FIG. 10 shows that the same results are obtained when only eosinophils present in the lung lavage are measured. Eosinophils are the type of inflammatory cell most closely associated with asthma.

FIG. 11 shows that when the mice are treated with a control oligo at the time of the initial exposure to the egg, there is little effect on the subsequent influx of eosinophils into the lungs after inhalation of SEA. Thus, when mice inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a CpG oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes the increase in eosinophils when the mice inhale the egg antigen on day 14.

FIG. 12 shows that very low doses of oligonucleotide (<10 μg) can give this protection.

FIG. 13 shows that the resultant inflammatory response correlates with the levels of the Th2 cytokine IL-4 in the lung.

FIG. 14 shows that administration of an oligonucleotide containing an unmethylated CpG motif can actually redirect

the cytokine response of the lung to production of Il-12, indicating a Th1 type of immune response.

FIG. 15 shows that administration of an oligonucleotide containing an unmethylated CpG motif can also redirect the cytokine response of the lung to production of IFN-γ, indicating a Th1 type of immune response.

Example 13

CpG Oligonucleotides Induce Human PBMC to Secrete Cytokines

Human PBMC were prepared from whole blood by standard centrifugation over ficoll hypaque. Cells ($5x10^5$/ml) were cultured in 10% autologous serum in 96 well microtiter plates with CpG or control oligodeoxynucleotides (24 μg/ml for phosphodiester oligonucleotides; 6 μ/ml for nuclease resistant phosphorothioate oligonucleotides) for 4 hr in the case of TNF-α or 24 hr. for the other cytokines before supernatant harvest and assay, measured by ELISA using Quantikine kits or reagents from R&D Systems (pg/ml) or cytokine ELISA kits from Biosource (for IL-12 assay). Assays were performed as per the manufacturer's instructions. Data are presented in Table 6 as the level of cytokine above that in wells with no added oligodeoxynucleotide.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 111

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGGAAGGTC CAGCGTTCTC 20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATCGACCTAC GTGCGTTCTC 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCCATAACGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs

-continued

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCTAGATGTT AGCGT 15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GAGAACGTCG ACCTTCGAT 19

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCATGACGTT GAGCT 15

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCCATGACGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCCATGAGCT TCCTGAGTCT 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCCAAGACGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TCCATGACGT TCCTGACGTT 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCCATGAGCT TCCTGAGTGC T 21

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGGGTCAACG TTGACGGGG 19

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 7...7
(D) OTHER INFORMATION: where N at position 7 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCTAGANGTT AGCGT 15

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 7...7
(D) OTHER INFORMATION: N at position 7 is 5 methyl cytosine
(A) NAME/KEY: misc_feature
(B) LOCATION: 13...13
(D) OTHER INFORMATION: N at position 13 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GCTAGANGTT AGNGT 15

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ATCGACTCTC GAGCGTTCTC 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3...3
(D) OTHER INFORMATION: where N at position 3 is 5 methyl cytosine
(A) NAME/KEY: misc_feature
(B) LOCATION: 10...10
(D) OTHER INFORMATION: where N at position 10 is 5 methyl cytosine
(A) NAME/KEY: misc_feature
(B) LOCATION: 14...14
(D) OTHER INFORMATION: where N at position 14 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ATNGACTCTN GAGNGTTCTC 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3...3
(D) OTHER INFORMATION: where N at position 3 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

-continued

ATNGACTCTC GAGCGTTCTC 20

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 18...18
(D) OTHER INFORMATION: where N at position 18 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

ATCGACTCTC GAGCGTTNTC 20

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ATGGAAGGTC CAACGTTCTC 20

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GAGAACGCTG GACCTTCCAT 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GAGAACGCTC GACCTTCCAT 20

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GAGAACGCTC GACCTTCGAT 20

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GAGCAAGCTG GACCTTCCAT 20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 6...6
(D) OTHER INFORMATION: where N at position 6 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GAGAANGCTG GACCTTCCAT 20

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 14...14
(D) OTHER INFORMATION: where N at position 14 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GAGAACGCTG GACNTTCCAT 20

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GAGAACGATG GACCTTCCAT 20

(2) INFORMATION FOR SEQ ID NO: 27:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GAGAACGCTC CAGCACTGAT 20

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

TCCATGTCGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TCCATGCTGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 8...8
(D) OTHER INFORMATION: where N at position 8 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

TCCATGTNGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 12...12
(D) OTHER INFORMATION: where N at position 12 is 5 methyl cytosine

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

TCCATGTCGG TNCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

TCCATGTCGG TCCTGCTGAT 20

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TCCATGCCGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

TCCATGGCGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

TCCATGACGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

TCCATGTCGA TCCTGATGCT 20

-continued

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

TCCATGTCGC TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

TCCATGTCGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

TCCATGACGT CCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

TCCATCACGT GCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

GGGGTCAGTC TTGACGGGG 19

(2) INFORMATION FOR SEQ ID NO: 42:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

GCTAGACGTT AGTGT 15

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 8...8
(D) OTHER INFORMATION: where N at position 8 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

GCTAGACNTT AGTGT 15

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 8...8
(D) OTHER INFORMATION: where N at position 8 is 5 methyl cytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

TCCATGTNGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

TCTCCCAGCG TGCGCCAT 18

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

TCGTCGTTTT GTCGTTTTGT CGTT 24

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

TCGTCGTTGT CGTTGTCGTT 20

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

TGTCGTTTGT CGTTTGTCGT T 21

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

TCGTCGTTGT CGTTTTGTCG TT 22

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

TGTCGTTGTC GTTGTCGTT 19

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

TCGTCGTCGT CGTT 14

-continued

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

TCCTGTCGTT CCTTGTCGTT 20

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

TCCTGTCGTT TTTTGTCGTT 20

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

TCGTCGCTGT CTGCCCTTCT T 21

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

TCGTCGCTGT TGTCGTTTCT T 21

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

GCGTGCGTTG TCGTTGTCGT T 21

(2) INFORMATION FOR SEQ ID NO: 57:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

GTCGTT 6

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

GTCGCT 6

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

ACCATGGACG ATCTGTTTCC CCTC 24

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

TACCGCGTGC GACCCTCT 18

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

ACCATGGACG AACTGTTTCC CCTC 24

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

ACCATGGACG AGCTGTTTCC CCTC 24

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

ACCATGGACG ACCTGTTTCC CCTC 24

(2) INFORMATION FOR SEQ ID NO: 64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:

ACCATGGACG TACTGTTTCC CCTC 24

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:

ACCATGGACG GTCTGTTTCC CCTC 24

(2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

ACCATGGACG TTCTGTTTCC CCTC 24

(2) INFORMATION FOR SEQ ID NO: 67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

CACGTTGAGG GGCAT 15

(2) INFORMATION FOR SEQ ID NO: 68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

CTGCTGAGAC TGGAG 15

(2) INFORMATION FOR SEQ ID NO: 69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

TCAGCGTGCG CC 12

(2) INFORMATION FOR SEQ ID NO: 70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70:

ATGACGTTCC TGACGTT 17

(2) INFORMATION FOR SEQ ID NO: 71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 71:

TCTCCCAGCG GGCGCAT 17

(2) INFORMATION FOR SEQ ID NO: 72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 72:

TCTCCCAGCG CGCGCCAT 18

-continued

(2) INFORMATION FOR SEQ ID NO: 73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73:

TCCATGTCGT TCCTGTCGTT 20

(2) INFORMATION FOR SEQ ID NO: 74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74:

TCCATAGCGT TCCTAGCGTT 20

(2) INFORMATION FOR SEQ ID NO: 75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

TCGTCGCTGT CTCCGCTTCT T 21

(2) INFORMATION FOR SEQ ID NO: 76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 76:

TCCTGACGTT CCTGACGTT 19

(2) INFORMATION FOR SEQ ID NO: 77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77:

TCCTGTCGTT CCTGTCGTT 19

(2) INFORMATION FOR SEQ ID NO: 78:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 78:

TCCATGTCGT TTTTGTCGTT 20

(2) INFORMATION FOR SEQ ID NO: 79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 79:

TCCAGGACTT CTCTCAGGTT 20

(2) INFORMATION FOR SEQ ID NO: 80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80:

TCCATGCGTG CGTGCGTTTT 20

(2) INFORMATION FOR SEQ ID NO: 81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 81:

TCCATGCGTT GCGTTGCGTT 20

(2) INFORMATION FOR SEQ ID NO: 82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 82:

TCCACGACGT TTTCGACGTT 20

(2) INFORMATION FOR SEQ ID NO: 83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 83:

GCGGCGGGCG GCGCGCGCCC 20

(2) INFORMATION FOR SEQ ID NO: 84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 84:

TGTCGTTGTC GTTGTCGTTG TCGTT 25

(2) INFORMATION FOR SEQ ID NO: 85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 85:

TGTCGTTGTC GTT 13

(2) INFORMATION FOR SEQ ID NO: 86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 86:

TCCACGACTT TTCGACGTT 19

(2) INFORMATION FOR SEQ ID NO: 87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87:

TCCATGACGA TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88:

-continued

TCCATAACGT CCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 89:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89:

TCCATGACGC TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 90:

GGGGTCAACG TTGAGGGGGG 20

(2) INFORMATION FOR SEQ ID NO: 91:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 91:

GCTAGACGTT AGCGT 15

(2) INFORMATION FOR SEQ ID NO: 92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 92:

GCTAGACGTT GAGCT 15

(2) INFORMATION FOR SEQ ID NO: 93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 93:

TCCATGACGT TCCTGCTGAT 20

-continued

(2) INFORMATION FOR SEQ ID NO: 94:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 94:

TCAACGTT 8

(2) INFORMATION FOR SEQ ID NO: 95:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 95:

TCAAGCTT 8

(2) INFORMATION FOR SEQ ID NO: 96:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 96:

TCAGCGCT 8

(2) INFORMATION FOR SEQ ID NO: 97:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 97:

TCATCGAT 8

(2) INFORMATION FOR SEQ ID NO: 98:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 98:

TCTTCGAA 8

(2) INFORMATION FOR SEQ ID NO: 99:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:8 base pairs

-continued

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 99:

CCAACGTT 8

(2) INFORMATION FOR SEQ ID NO: 100:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 100:

TCAACGTC 8

(2) INFORMATION FOR SEQ ID NO: 101:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 101:

TCCATGGTGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 102:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 102:

TCCATGGTGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 103:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 103:

TCCATGATAG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 104:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 104:

TCCATGATCG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 105:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 105:

TCCATGATTG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 106:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 106:

TCCATGATTG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO: 107:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 107:

TCCAGGACTT TCCTCAGGTT 20

(2) INFORMATION FOR SEQ ID NO: 108:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 108:

TCCAGGACTT TCCTCAGGTT 20

(2) INFORMATION FOR SEQ ID NO: 109:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 109:

-continued

GGCGTTATTC CTGACTCGCC 20

(2) INFORMATION FOR SEQ ID NO: 110:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 110:

CCTACGTTGT ATGCGCCCAG CT 22

(2) INFORMATION FOR SEQ ID NO: 111:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 111:

TGTCGCT 7

We claim:

1. A method for inducing Il-6 in a subject comprising administering to the subject an effective amount to induce Il-6 in the subject of an immunostimulatory nucleic acid, having a sequence comprising:

5'$X_1X_2CGX_3X3$'

wherein C is unmethylated, wherein X_1, X_2 and X_3, X_4 are nucleotides, and wherein the 5' X_1 X_2CGX_3 X_4 3' sequence is a non-palindromic sequence.

2. The method of claim 1, wherein the subject is human.

3. The method of claim 1, wherein the nucleic acid has 8 to 100 nucleotides.

4. The method of claim 1, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

5. The method of claim 1, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

6. The method of claim 1, wherein the nucleic acid includes a phosphate backbone modification.

7. The method of claim 1, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, GpT, GpA, CpG, TpA, TpT, and TpG; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA.

8. The method of claim 1, wherein X_1X_2 are GpA and X_3X_4 are TpT.

9. The method of claim 1, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

10. The method of claim 1, wherein X_1X_2 are GpA and X_3 and X_4 are pyrimidines.

11. The method of claim 1, wherein the immunostimulatory nucleic acid is 8 to 40 nucleotides in length.

12. The method of claim 1, wherein the immunostimulatory nucleic acid, has a sequence comprising:

5'$NX_1X_2CGX_3X_4N3$'

wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2–25 nucleotides.

13. The method of claim 1, wherein the immunostimulatory nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATAACGTTCCTGATGCT (SEQ ID NO:2); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGGCGGTCCTGATGCT (SEQ ID NO:34); TCCATGTCGGTCCTGATGCT (SEQ ID NO:28); TCCATAACGTCCCTGATGCT (SEQ ID NO:88); TCCATGTCGTTCCTGATGCT (SEQ ID NO:38); and TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46).

14. A method of stimulating natural killer cell lytic activity comprising exposing a natural killer cell to an immunostimulatory nucleic acid to stimulate natural killer cell lytic activity, the immunostimulatory nucleic acid having a sequence comprising:

5'$X_1X_2CGX_3X3$'

wherein C is unmethylated, wherein X_1X_2 and X_3X_4 are nucleotides, and wherein the 5'$X_1X_2CGX_3$ $X_4$3' sequence is a non-palindromic sequence.

15. The method of claim 14, wherein the nucleic acid has 8 to 100 nucleotides.

16. The method of claim 14, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

17. The method of claim 14, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

18. The method of claim 14, wherein the nucleic acid includes a phosphate backbone modification.

19. The method of claim 14, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA.

20. The method of claim 14, wherein X_1X_2 are GpA and X_3X_4 are TpT.

21. The method of claim 14, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

22. The method of claim 14, wherein X_1X_2 are GpA and X_3 and X_4 are pyrimidines.

23. The method of claim 14, wherein the immunostimulatory nucleic acid is 8 to 40 nucleotides in length.

24. The method of claim 15, wherein the immunostimulatory nucleic acid, has a sequence comprising:

5'$NX_1X_2CGX_3X_4N$3'

wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2–25 nucleotides.

25. The method of claim 14, wherein the immunostimulatory nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:47); TCCATGACGGTCCTGATGCT (SEQ ID NO:35); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGACGCTCCTGATGCT (SEQ ID NO:89); TCCATGACGTTCCTGATGCT (SEQ ID NO:7); TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46); TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:49); GCGTGCGTTGTCGTTGTCGTT (SEQ ID NO:56); TGTCGTTTGTCGTTTGTCGTT(SEQ ID NO:48); TGTCGTTGTCGTTGTCGTT (SEQ ID NO:50); and TCGTCGTCGTCGTT (SEQ ID NO:51).

26. A method for inducing interferon-gamma in a subject to treat an immune system deficiency, comprising:

administering to a subject having an immune system deficiency an effective amount to induce interferon-gamma production in the subject of an immunostimulatory nucleic acid, having a sequence comprising:

5'$X_1X_2CGX_3X_4$3'

wherein C is unmethylated, wherein X_1X_2 and X_3X_4 are nucleotides, and wherein the sequence of the formula $X_1X_2CGX_3X_4$ is not palindromic.

27. The method of claim 26, wherein the nucleic acid has 8 to 100 nucleotides.

28. The method of claim 26, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

29. The method of claim 26, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

30. The method of claim 26, wherein the nucleic acid includes a phosphates backbone modification.

31. The method of claim 26, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA.

32. The method of claim 26, wherein X_1X_2 are GpA and X_3X_4 are TpT.

33. The method of claim 26, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

34. The method of claim 26, wherein X_1X_2 are GpA and X_3 and X_4 are pyrimidines.

35. The method of claim 26, wherein the immunostimulatory nucleic acid is 8 to 40 nucleotides in length.

36. The method of claim 26, wherein the immunostimulatory nucleic acid, has a sequence comprising:

5'$NX_1X_2CGX_3X_4N$3'

wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2–25 nucleotides.

37. A method for inducing Il-12 in a subject comprising:

administering to the subject an effective amount to induce Il-12 in the subject, of an immunostimulatory nucleic acid having a sequence comprising:

5'$X_1X_2CGX_3X$3'

wherein C is unmethylated, wherein X_1, X_2, X_3, and X_4 are nucleotides, and wherein the sequence of the formula $X_1X_2CGX_3X_4$ is not palindromic.

38. The method of claim 37, wherein the subject is human.

39. The method of claim 37, wherein the immunostimulatory nucleic acid sequence is selected from the group consisting of sequences comprising the following nucleotides: TCCATGTCGCTCCTGATGCT (SEQ ID NO:37); TCCATGACGATCCTGATGCT (SEQ ID NO:87); TCCATGGCGGTCCTGATGCT (SEQ ID NO:34); TCCATGTCGGTCCTGATGCT (SEQ ID NO:28); TCCATAACGTCCCTGATGCT (SEQ ID NO:88); TCCATGTCGTTCCTGATGCT (SEQ ID NO:38); and TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:46).

40. The method of claim 37, wherein the nucleic acid has 8 to 100 nucleotides.

41. The method of claim 37, wherein the nucleic acid backbone includes a phosphate backbone modification on the 5' inter-nucleotide linkages.

42. The method of claim 37, wherein the nucleic acid backbone includes a phosphate backbone modification on the 3' inter-nucleotide linkages.

43. The method of claim 37, wherein the nucleic acid includes a phosphate backbone modification.

44. The method of claim 37, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA.

45. The method of claim 37, wherein X_1X_2 are GpA and X_3X_4 are TpT.

46. The method of claim 37, wherein X_1 and X_2 are purines and X_3 and X_4 are pyrimidines.

47. The method of claim 37, wherein X_1X_2 are GpA and X_3 and X_4 are pyrimidines.

48. The method of claim 37, wherein the immunostimulatory nucleic acid is 8 to 40 nucleotides in length.

49. The method of claim 37, wherein the immunostimulatory nucleic acid, has a sequence comprising:

5'$NX_1X_2CGX_3X_4N$3'

wherein X_1, X_2, X_3, and X_4 are nucleotides and N is a nucleic acid sequence composed of from about 2–25 nucleotides.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,239,116 B1
DATED : May 29, 2001
INVENTOR(S) : Krieg et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [75], Inventors: **Arthur M. Krieg**, Iowa City, IA (US); **Dennis Klinman**, Potomac, MD (US); and **Alfred D. Steinberg**, Potomac, MD (US) delete "**Joel N. Kline**, Iowa City, IA (US)"
Item [57], **ABSTRACT**,
Line 5, after the word "useful" insert -- as --.

Column 3,
Line 5, delete "(dG).(dC)" and insert -- (dG)·(dC) --.
Line 5, delete "(dG.dC)" and insert -- (dG·dC) --.
Line 10, delete "dG.dC" and insert -- dG·dC --.
Line 11, after the word "Tokunaga," insert -- T., --.
Line 12, delete "α/b" and insert -- α/β --.
Line 13, delete "-g" and insert -- -γ --.
Line 32, delete "0." and insert -- O. --.
Line 39, delete "Ig2b" and insert -- Iγ2b --
Line 40, delete "g2b" and insert -- γ2b --.

Column 4,
Line 18, after "H." insert -- , --.
Line 26, after "1" insert -- β --.
Line 32, delete "B" and insert -- β --.
Line 36, delete "TGF-B1" and insert -- TGF-β1 --.
Line 39, delete "DRa" and insert -- DRα --.

Column 5,
Line 26, after the word "functions" delete ";".
Lines 41, 43, 45, 47 and 49, delete "E1A" and insert -- E1a --.

Column 6,
Line 34, after the word "that" delete "that".

Column 8,
Line 3, delete "5'CCATGACGTTCCTGATGCT3'" and insert -- 5'TCCATGACGTTCCTGATGCT3' --.

Column 9,
Line 40, delete "IFN-g" and insert -- IFN-γ --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 11,
Line 30, delete "immunostimulalory" and insert -- immunostimulatory --.
Line 34, delete "5'$N_zX_1CGX_2N_23$'" and insert -- 5'$N_1X_1CGX_2N_23$' --.
Line 52, after the word "that" delete "that".
Line 57, delete "invnetion" and insert -- invention --.
Line 67, after the word "a" delete "a".

Column 12,
Line 31, delete "TCGTCGTTTTGTCGTTTTGTCGT" and insert
-- TCGTCGTTTTGTCGTTTTGTCGTT --.
Line 37, delete "TCGTCGTTTTGTCGTTTGTCGTT" and insert
-- TCGTCGTTTTGTCGTTTTGTCGTT --.

Column 13,
Line 53, delete "ABCDEED'C'B'A'" and insert -- ABCDEE'D'C'B'A' --.

Column 16,
Line 19, delete "id" and insert -- 1d --.
Lines 37 and 39, after the word "ends" delete ",".
Table 1, delete "Olionucleotide" and insert -- Oligonucleotide --.

Column 17,
Table 1, delete "Olionucleotide" and insert -- Oligonucleotide --.
Table 1, after the No. "3Da"(SEQ ID NO:21), delete "..... C....." and insert
-- ..... C..... --.

Column 20,
Line 35, delete ":" and insert -- , " --.

Column 21,
Line 23, delete "nonresponseive" and insert -- nonresponsive --.

Column 22,
Line 24, delete "harbor" and insert -- Harbor --.
Line 45, delete "lipopolysaccaride" and insert -- lipopolysaccharide --.
Line 59, after the word "activating" delete ";".

Column 24,
Line 17, delete "moths" and insert -- motifs --.
Table 5, delete "secrtetion" and insert -- secretion --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,239,116 B1
DATED : May 29, 2001
INVENTOR(S) : Krieg et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 25,
Table 5, delete "secrtetion" and insert -- secretion --.
Table 5, delete "$_1$" and insert -- ^{1} --.
Line 39, after the word "residues" insert -- 6-11 --.
Line 50, delete "8" and insert -- 88 --.

Column 26,
Line 54, after the word "human" delete ",".

Column 27,
Line 15, after the word "IL-" insert -- 6 --.
Line 16, delete "6promoter" and insert -- promoter --.

Column 28,
Line 56, delete "All" and insert -- all --.

Column 30,
Line 27, after the word "NK" delete ";".
Line 29, delete "α/b" and insert -- α/β --.
Table 9, after the number "1629" delete "-------gtc----(SEQ ID NO:50)" and insert -- ------gtc--------(SEQ ID NO:41) --.
Table 9, after the number "1765" delete "-------Z--------(SEQ ID NO:53)" and insert -- ------Z----------(SEQ ID NO:44) --.

Column 31,
Line 2, after the word "immune" delete ",".

Column 33,
Table 11, delete "Phoshorothioate" and insert -- Phosphorothioate --.
Table 11, after the number "1982" delete "TCCAGGACTTCTCTCAAGTT" and insert -- TCCAGGACTTCTCTCAGGTT --.

Columns 33-34,
Table 12, in the heading, delete "Index ^{1L}" and insert -- Index^1 --.

Columns 35-36,
Table 12, in the heading, delete "Index ^{1L}" and insert -- Index^1 --.

Column 35,
Line 65, delete "IL2" and insert -- IL 12 --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,239,116 B1
DATED : May 29, 2001
INVENTOR(S) : Krieg et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 37,
Line 54, after the word "Immunostimulatory" delete ",".
Line 60, delete "2nd" and insert -- and --.

Column 39,
Line 8, delete "sis" and insert -- as --.

Column 41,
Line 24, delete "oiler" and insert -- other --.

Columns 41-42,
Table 15, in the line beginning with "TABLE 15", delete "3774" and insert -- J774 --.
Table 15, after the word "(TNF-α" insert --) --.

Column 43,
Line 55, delete "Coμparable aμounts" and insert -- Comparable amounts --.
Line 56, delete "ribosoμal μDNA" and insert -- ribosomal mDNA --.
Line 57, delete "tides" and insert -- times --.

Column 46,
Line 19, after the word "acids" delete ",".
Line 47, delete "unethylated" and insert -- unmethylated --.
Line 48, delete "TCCATGACGTTCCTGACGTT" and insert
-- TCCATGACGTTCCTGACGTT --.
Line 63, delete "pareniteral" and insert -- parenteral --.

Column 48,
Line 39, delete "37 C" and insert -- 37° C. --.
Line 40, delete "al5so" and insert -- also --.
Line 58, after the word "washed" delete ",".
Line 65, after the word "centrifuged" delete ",".

Column 49,
Line 9, delete "phophorothioate" and insert -- phosphorothioate --.
Line 31, after the number "37" insert -- ° --.
Line 51, delete "Elisa" and insert -- ELISA --.

Column 50,
Line 4, delete "Homby" and insert -- Hornby --.
Line 62, delete "$NaHC_3$" and insert -- $NaHCO_3$ --.
Line 64, after the number "6.6 mM" delete ",".

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,239,116 B1
DATED : May 29, 2001
INVENTOR(S) : Krieg et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 51,
Line 26, delete "Dalhman" and insert -- Dallman --.
Line 39, delete "(tried" and insert -- dried --.
Line 56, delete "electroprated" and insert -- electroporated --.

Column 52,
Line 22, delete "benzoyhnercaptoethyl" and insert -- benzoylmercaptoethyl --.
Line 39, after the word "kinetics" delete ",".

Column 53,
Line 1, delete "(5" GAGAACCTGGACCTTCCAT)" and insert
-- (5' GAGAACGCTGGACCTTCCAT) --.
Line 3, delete "(5' TCCATGTCGTCCTGATGCT)" and insert
-- (5' TCCATGTCGGTCCTGATGCT) --.
Line 5, delete "(5' GGCGTTATTCCTGACTCGCC)" and insert
-- (5' GGCGTTATTCCTGACTCGCC) --.
Line 7, delete "(5' CCTACGTTGTATGCGCCCAGCT)" and insert
-- (5' CCTACGTTGTATGCGCCCAGCT) --.
Line 52, before the word "PBMC)" insert -- (--.

Column 54,
Lines 26-27, delete "TCCATGACGTTCCTGACGTT" and insert
-- TCCATGACGTTCCTGACGTT --.

Column 55,
Line 1, delete "Il-12" and insert -- IL-12 --.

Column 99,
Line 38, delete "5'$X_1X_2CGX_3X3$'" and insert -- 5'$X_1X_2CGX_3X_4$3' --.
Line 54, delete "GpT, GpA," and insert -- CpT, CpA, --.

Column 100,
Line 53, delete "5'$X_1X_2CGX_3X3$'" and insert -- 5'$X_1X_2CGX_3X_4$3' --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,239,116 B1
DATED : May 29, 2001
INVENTOR(S) : Krieg et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 101,
Line 14, delete "15" and insert -- 14 --.
Line 57, delete "phosphates" and insert -- phosphate --.
Line 19, delete "5'$X_1X_2CGX_3X3$'" and insert -- 5'$X_1X_2CGX_3X_43$' --.

Signed and Sealed this

Tenth Day of June, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

US006406705B1

(12) **United States Patent**
Davis et al.

(10) **Patent No.: US 6,406,705 B1**
(45) **Date of Patent: Jun. 18, 2002**

(54) **USE OF NUCLEIC ACIDS CONTAINING UNMETHYLATED CPG DINUCLEOTIDE AS AN ADJUVANT**

(75) Inventors: **Heather L. Davis**, Ottawa (CA); **Joachim Schorr**, Hilden (DE); **Arthur M. Krieg**, Iowa City, IA (US)

(73) Assignees: **University of Iowa Research Foundation**, Iowa City, IA (US); **Coley Pharmaceutical GmbH**, Langenfield (DE); **Ottawa Health Research Institute**, Ottawa (CA)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/325,193**

(22) Filed: **Jun. 3, 1999**

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/154,614, filed as application No. PCT/US98/04703 on Mar. 10, 1998.
(60) Provisional application No. 60/040,376, filed on Mar. 10, 1997.

(51) **Int. Cl.**[7] **A61K 45/00**; A61K 39/12
(52) **U.S. Cl.** **424/278.1**; 424/279.1; 424/282.1; 424/204.1; 536/23.72; 930/200; 930/210; 930/220
(58) **Field of Search** 424/278.1, 279.1, 424/282.1, 204.1; 930/200, 210, 220; 536/23.72

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(List continued on next page.)

Primary Examiner—Ali R. Salimi
(74) *Attorney, Agent, or Firm*—Wolf, Greenfield & Sacks, P.C.

(57) **ABSTRACT**

The present invention relates generally to adjuvants, and in particular to methods and products utilizing a synergistic combination of immunostimulatory oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) and a non-nucleic acid adjuvant. Such combinations of adjuvants may be used with an antigen or alone. The present invention also relates to methods and products utilizing immunostimulatory oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) for induction of cellular immunity in infants.

11 Claims, 12 Drawing Sheets

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FIG. 1

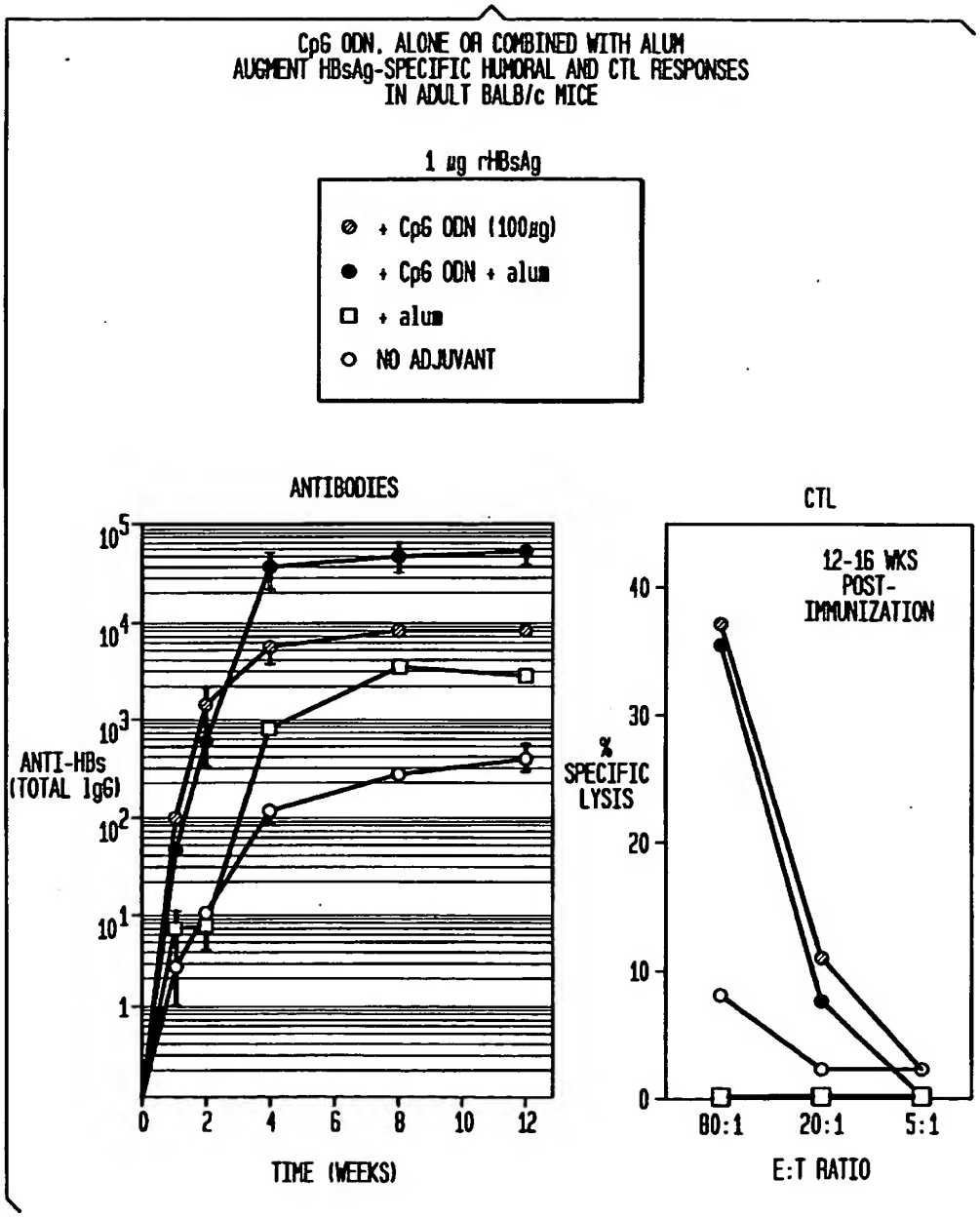

CpG ODN, ALONE OR COMBINED WITH ALUM
AUGMENT HBsAg-SPECIFIC HUMORAL AND CTL RESPONSES
IN ADULT BALB/c MICE
1 μg rHBsAg
+ CpG ODN (100μg)
+ CpG ODN + alum
+ alum
NO ADJUVANT
ANTIBODIES
ANTI-HBs (TOTAL IgG)
10^5
10^4
10^3
10^2
10^1
1
0 2 4 6 8 10 12
TIME (WEEKS)
CTL
12-16 WKS POST-IMMUNIZATION
% SPECIFIC LYSIS
40
30
20
10
0
80:1 20:1 5:1
E:T RATIO

FIG. 2

IMMUNOSTIMULATORY CpG ODN AS AN ADJUVANT FOR HBV VACCINE: EFFECT OF DOSE IN BALB/c MICE

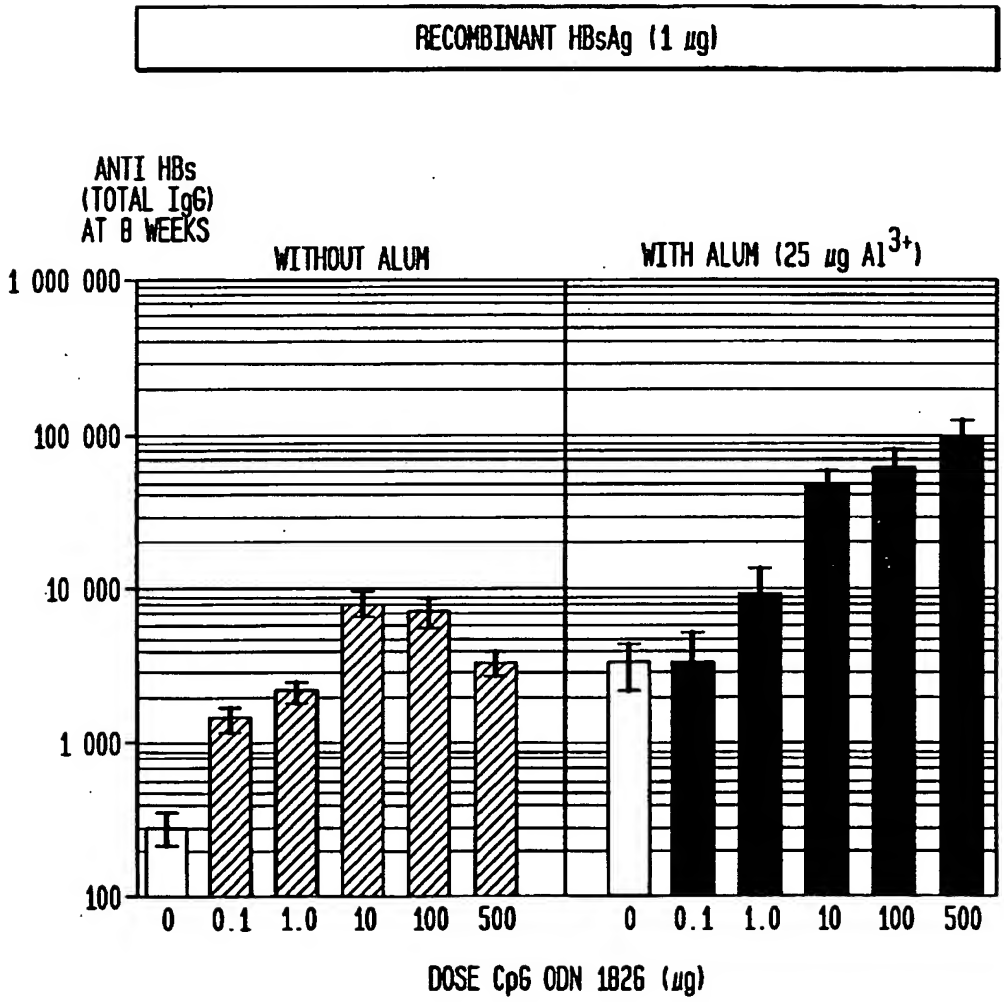


FIG. 3

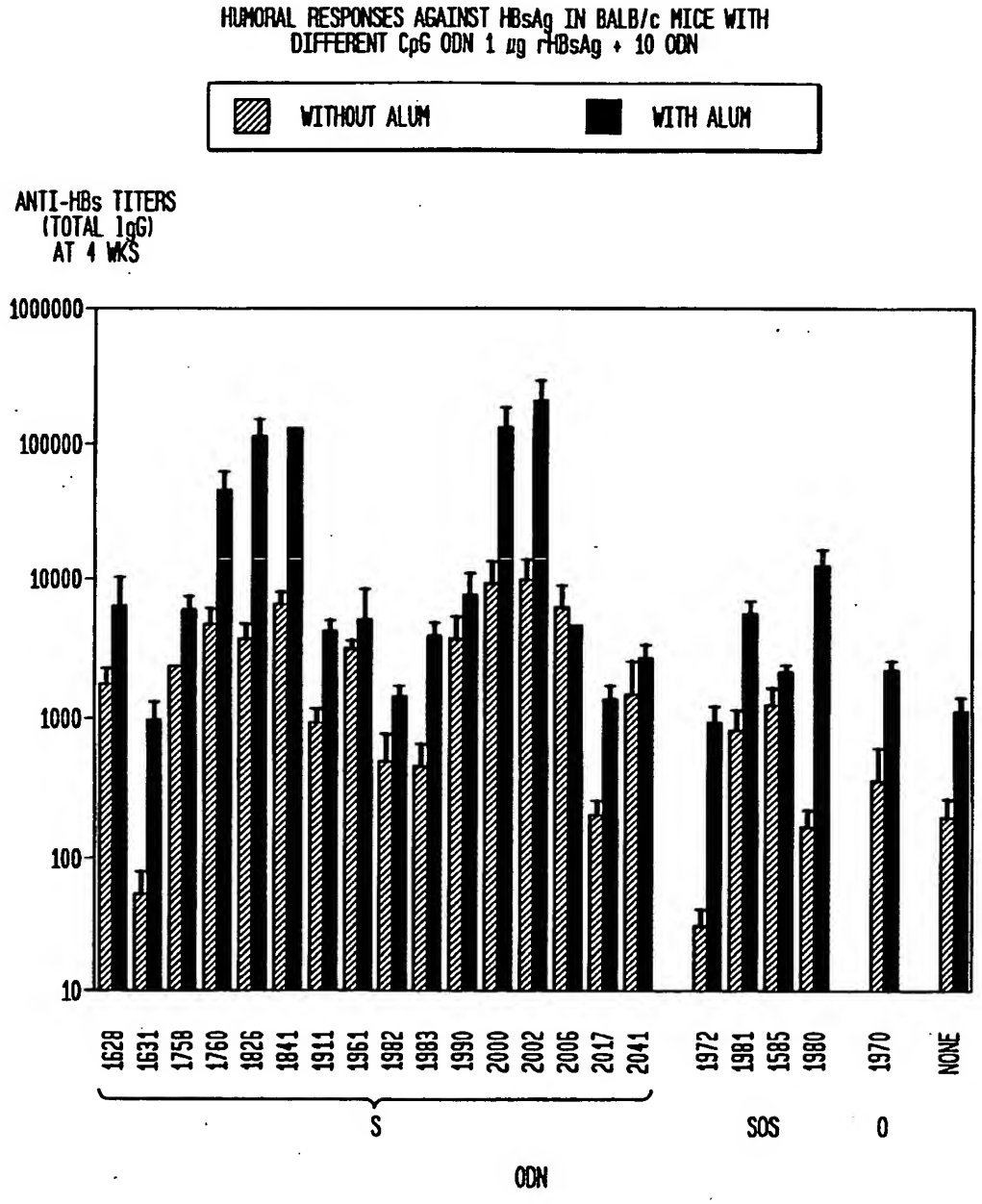

HUMORAL RESPONSES AGAINST HBsAg IN BALB/c MICE WITH DIFFERENT CpG ODN 1 μg rHBsAg + 10 ODN
WITHOUT ALUM
WITH ALUM
ANTI-HBs TITERS (TOTAL IgG) AT 4 WKS
1000000
100000
10000
1000
100
10
1628
1631
1758
1760
1826
1841
1911
1961
1982
1983
1990
2000
2002
2006
2017
2041
1972
1981
1585
1980
1970
NONE
S
SOS
O
ODN

FIG. 4

CTL RESPONSES IN BALB/c MICE WITH CpG ODN AND/OR ALUM AS ADJUVANT IN PRIME-BOOST STRATEGIES

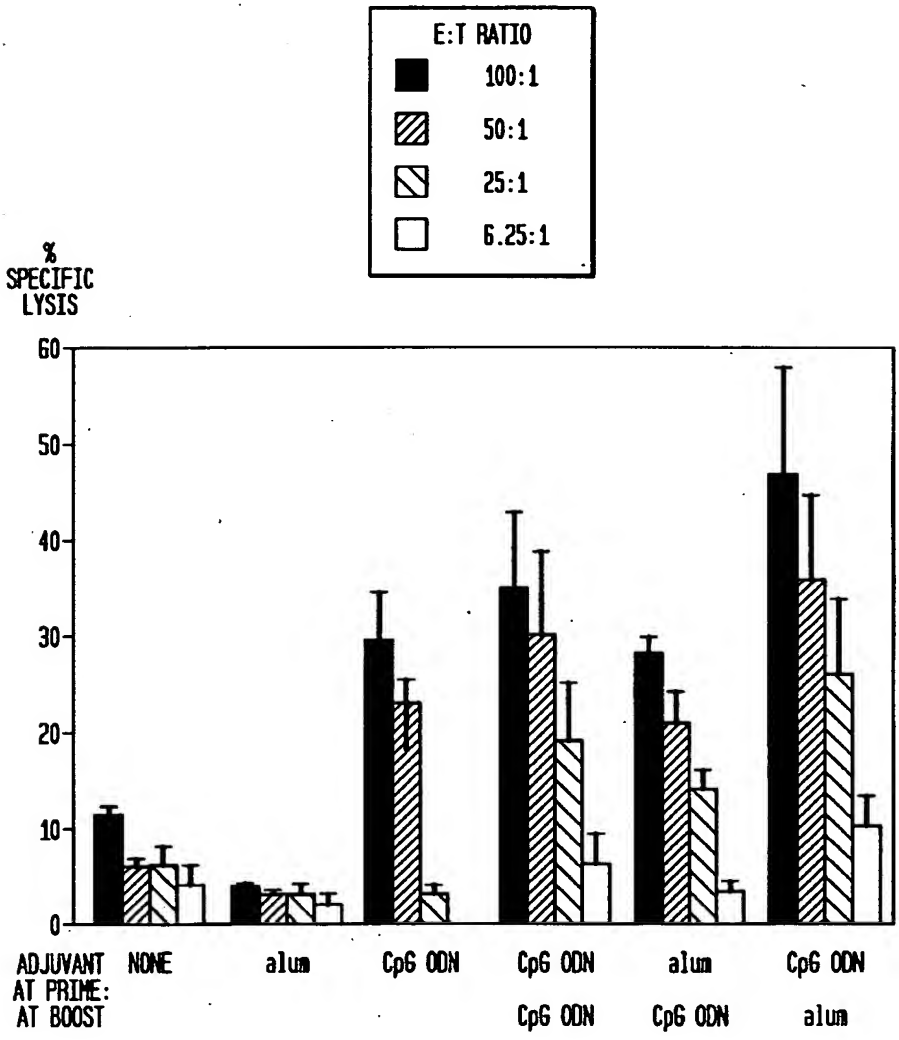


FIG. 5

COMPARISON OF DIFFERENT ADJUVANTS FOR IMMUNIZATION OF BALB/c MICE AGAINST rHBsAg

HBsAg (1μg) +	
ALUM	25 μg
CpG ODN	10 μg
MPL	50 μg
FCA	1:1 v/v

NUMBERS ABOVE BARS ARE RATIO OF IgG2a:IgG1 WHERE A NUMBER > 1 INDICATES A Th1-TYPE RESPONSE

NUMBERS ON BARS ARE NUMBER-FOLD INCREASE OVER HBsAg ALONE FOR ANTI-HBs TOTAL IgG

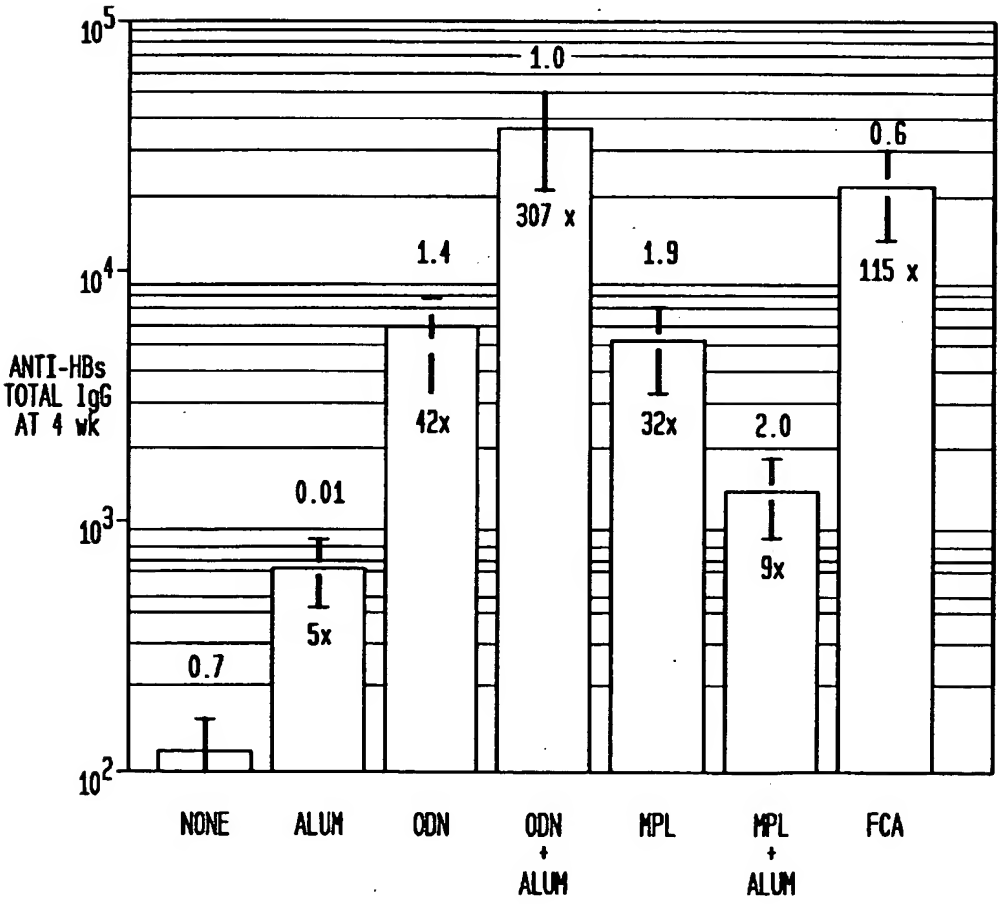
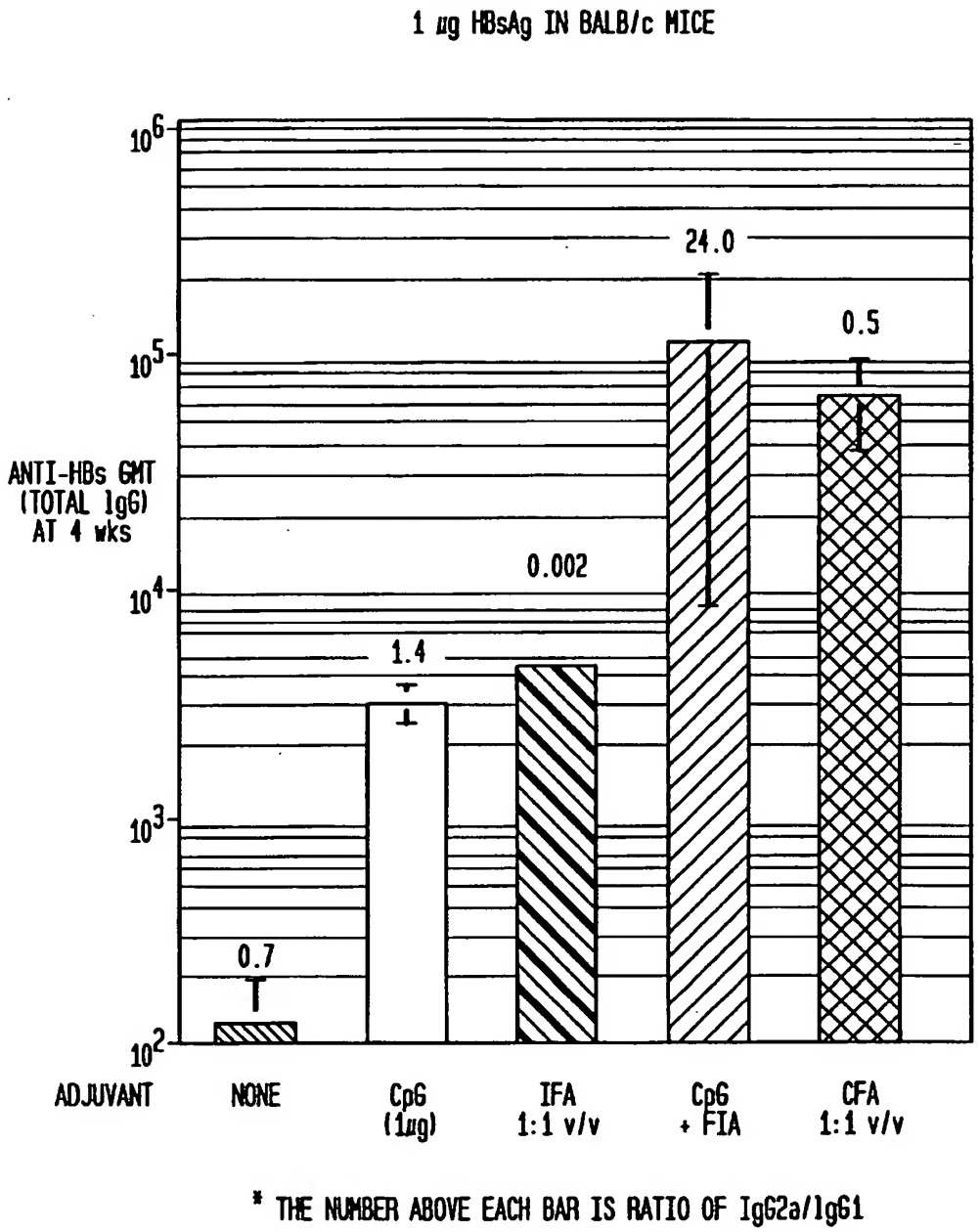
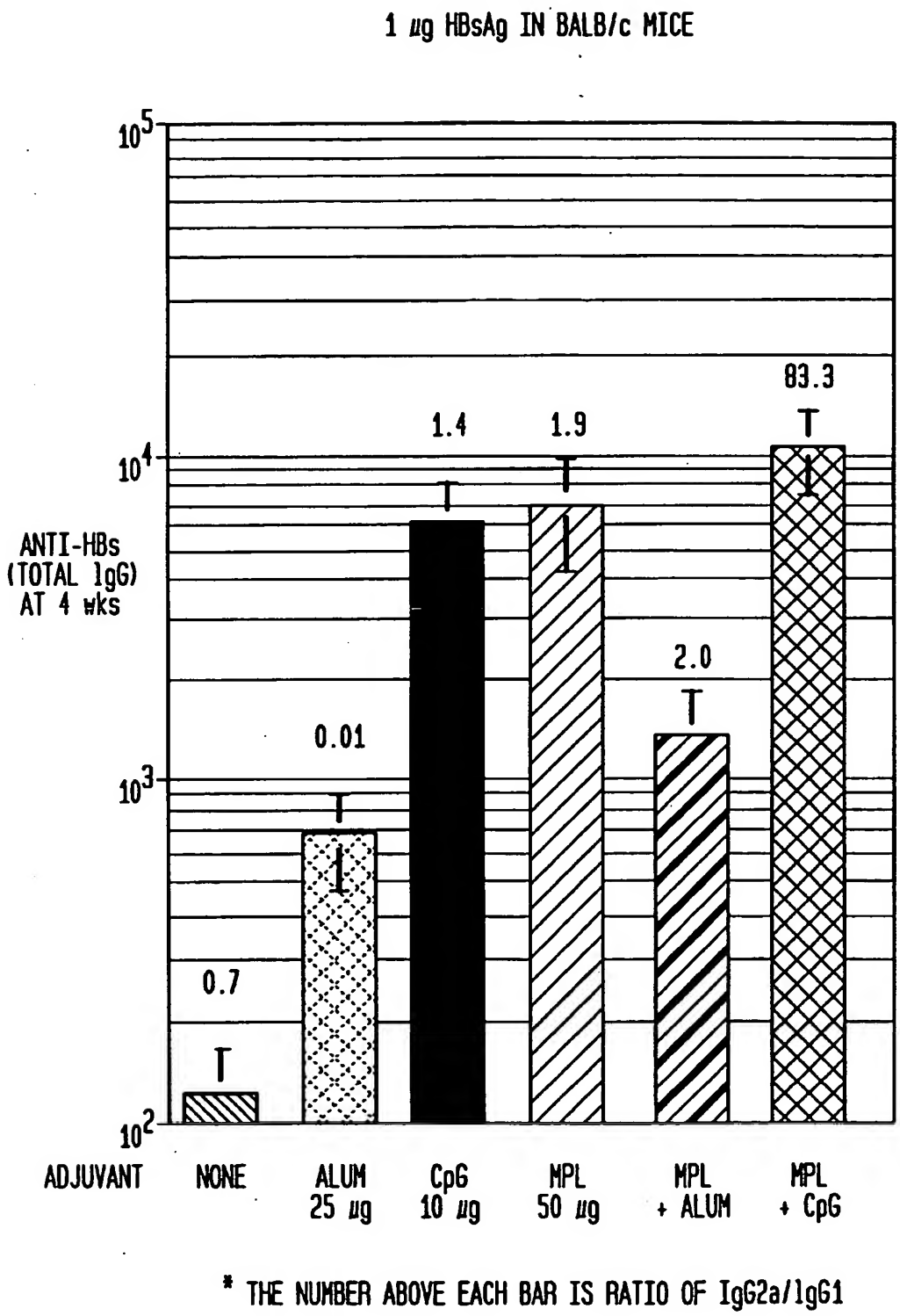


FIG. 6



* THE NUMBER ABOVE EACH BAR IS RATIO OF IgG2a/IgG1

FIG. 7



* THE NUMBER ABOVE EACH BAR IS RATIO OF IgG2a/IgG1

FIG. 8

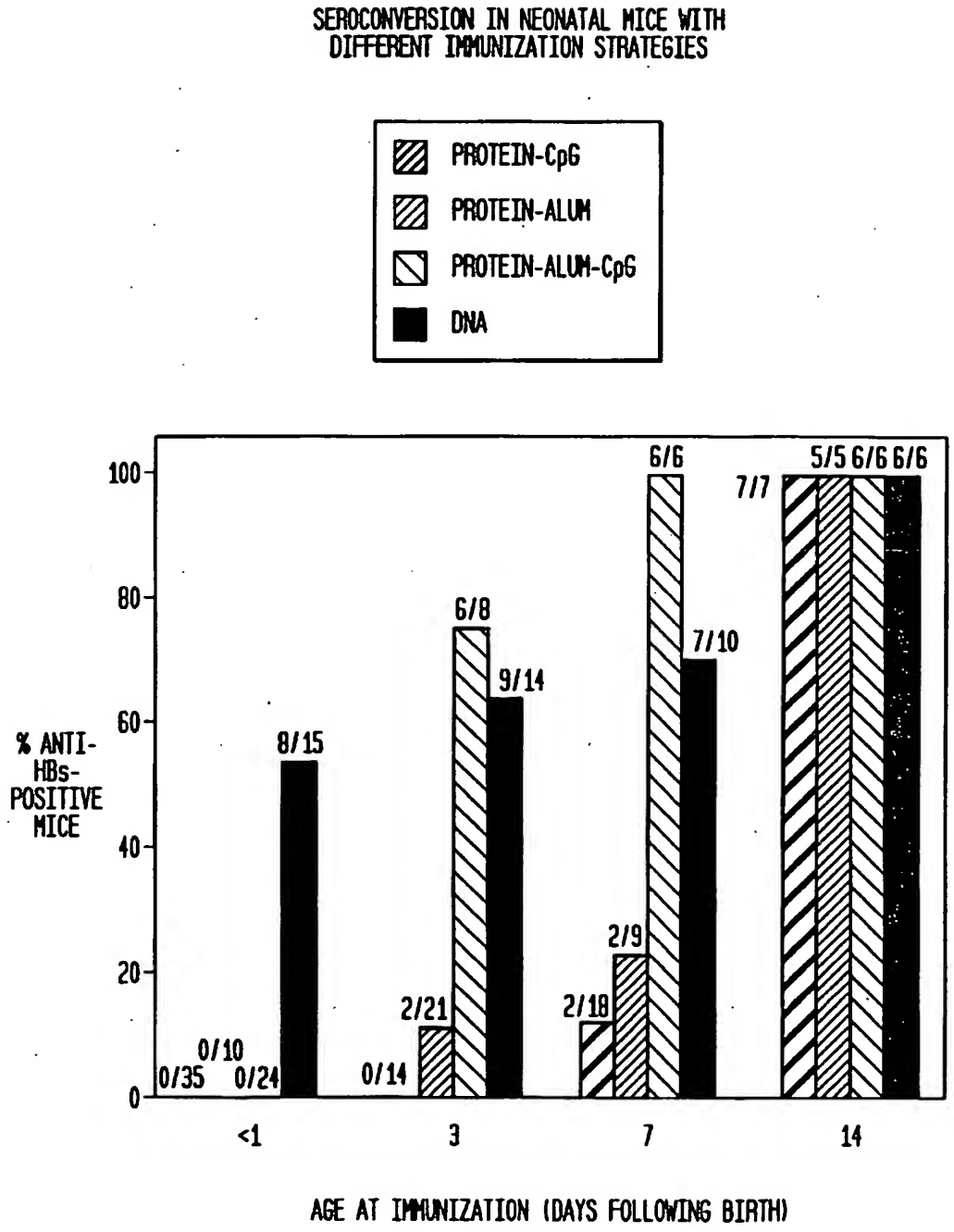

SEROCONVERSION IN NEONATAL MICE WITH DIFFERENT IMMUNIZATION STRATEGIES
PROTEIN-CpG
PROTEIN-ALUM
PROTEIN-ALUM-CpG
DNA
% ANTI-HBs-POSITIVE MICE
100
80
60
40
20
0
0/35
0/10
0/24
8/15
0/14
2/21
6/8
9/14
2/18
2/9
6/6
7/10
7/7
5/5
6/6
6/6
<1
3
7
14
AGE AT IMMUNIZATION (DAYS FOLLOWING BIRTH)

FIG. 9

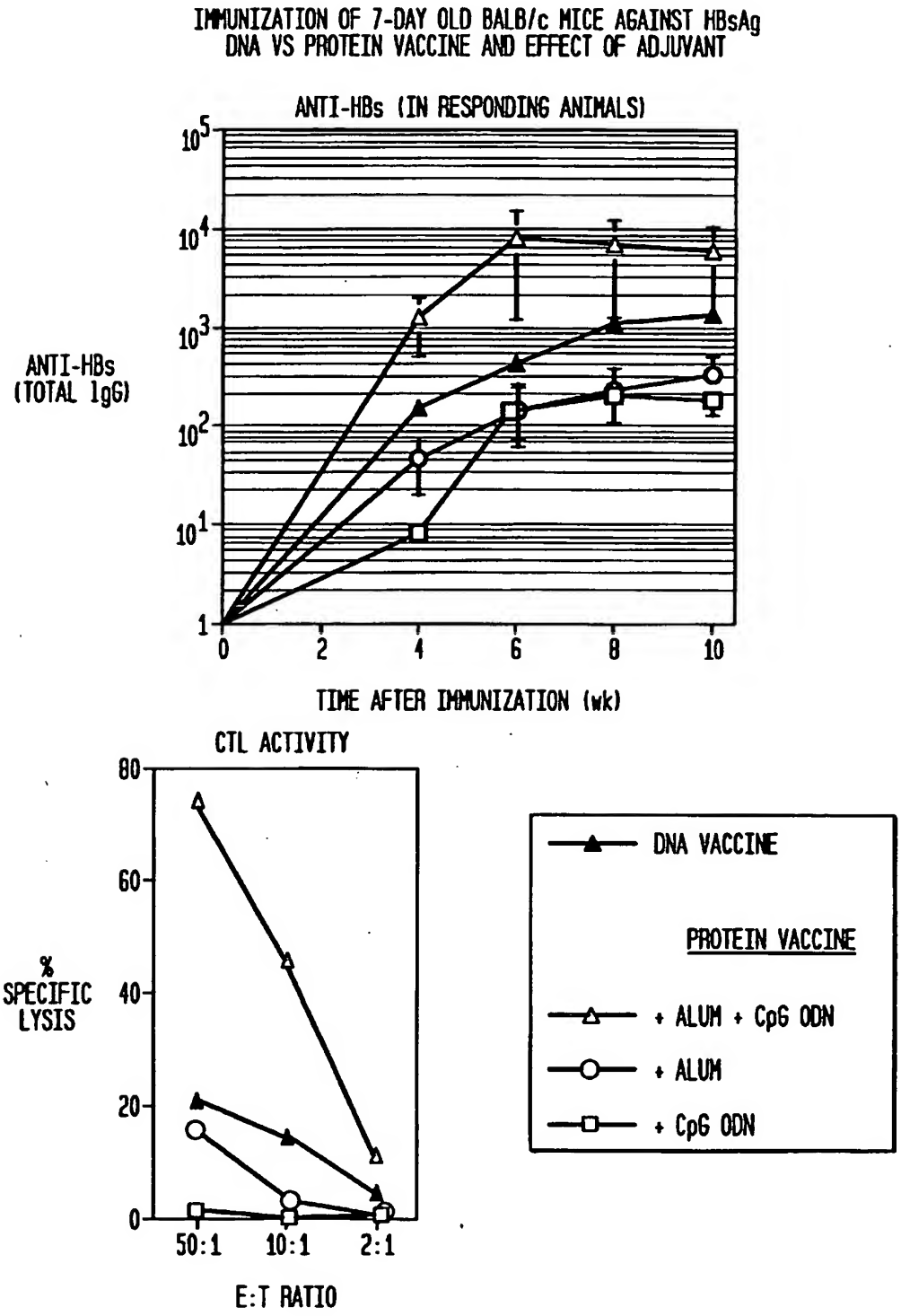

IMMUNIZATION OF 7-DAY OLD BALB/c MICE AGAINST HBsAg
DNA VS PROTEIN VACCINE AND EFFECT OF ADJUVANT
ANTI-HBs (IN RESPONDING ANIMALS)
ANTI-HBs (TOTAL IgG)
10^5
10^4
10^3
10^2
10^1
1
0
2
4
6
8
10
TIME AFTER IMMUNIZATION (wk)
CTL ACTIVITY
% SPECIFIC LYSIS
80
60
40
20
0
50:1
10:1
2:1
E:T RATIO
DNA VACCINE
PROTEIN VACCINE
+ ALUM + CpG ODN
+ ALUM
+ CpG ODN

FIG. 10

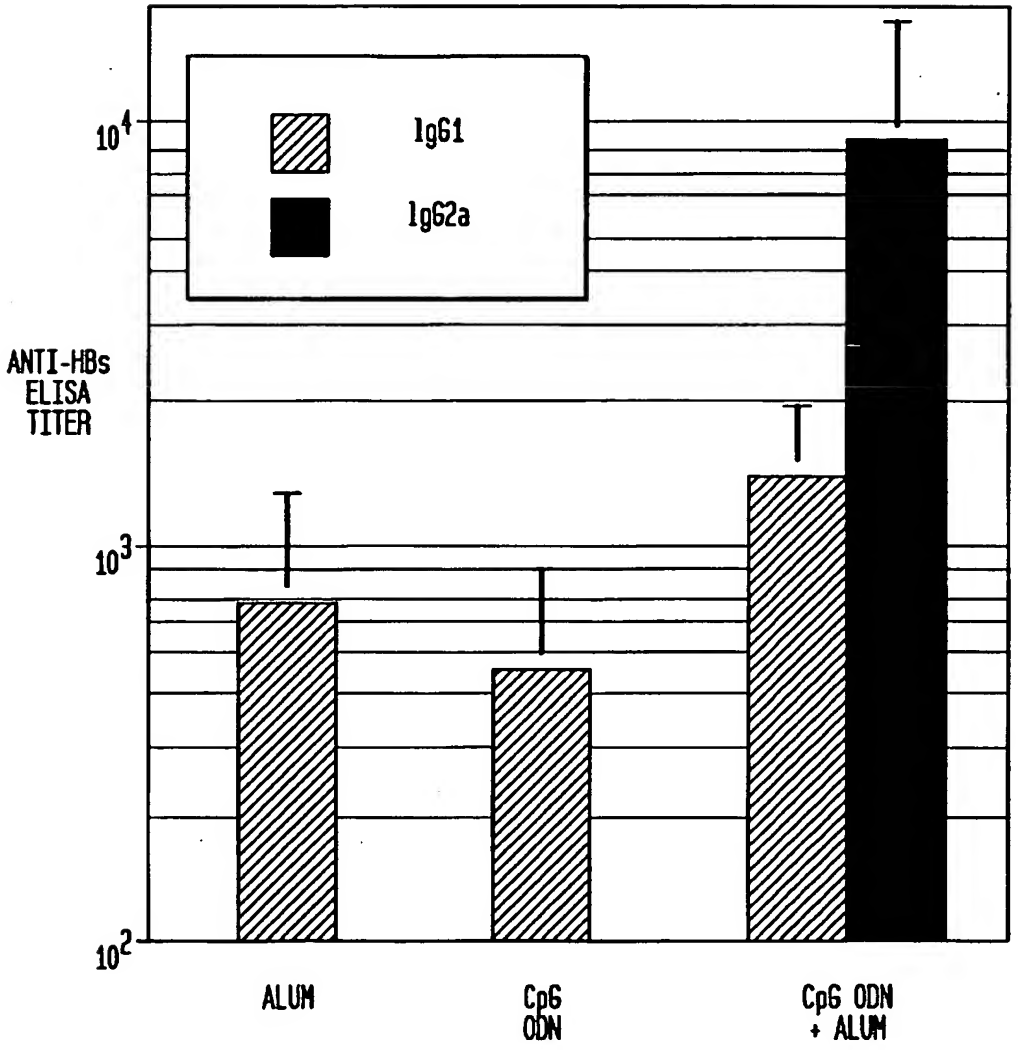

DNA VS PROTEIN IMMUNIZATION IN
NEONATAL (7 DAY OLD) BALB/c MICE
EFFECT OF ADJUVANT ON AB ISOTOPE
IgG1
IgG2a
ANTI-HBs
ELISA
TITER
10^4
10^3
10^2
ALUM
CpG
ODN
CpG ODN
+ ALUM

FIG. 11

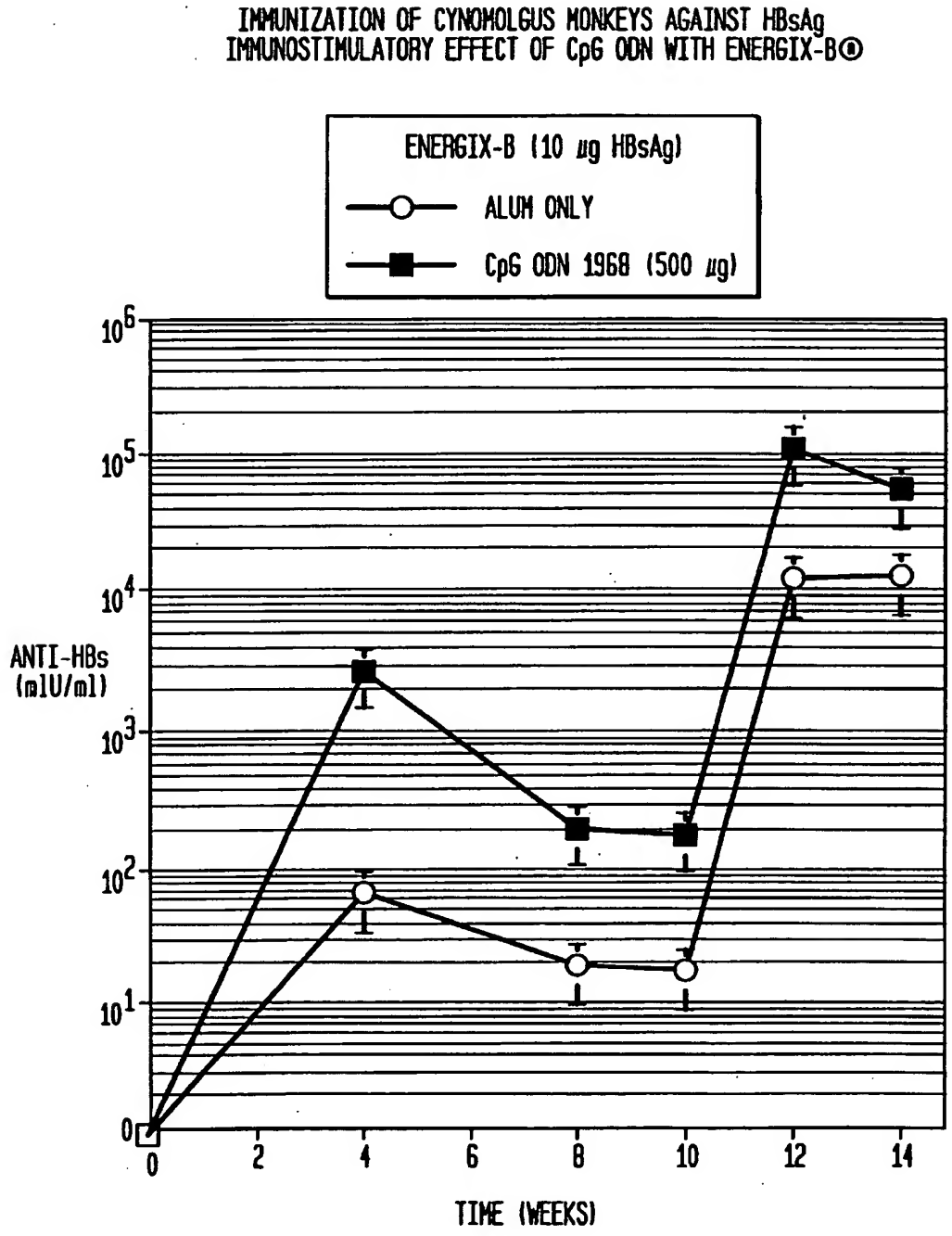
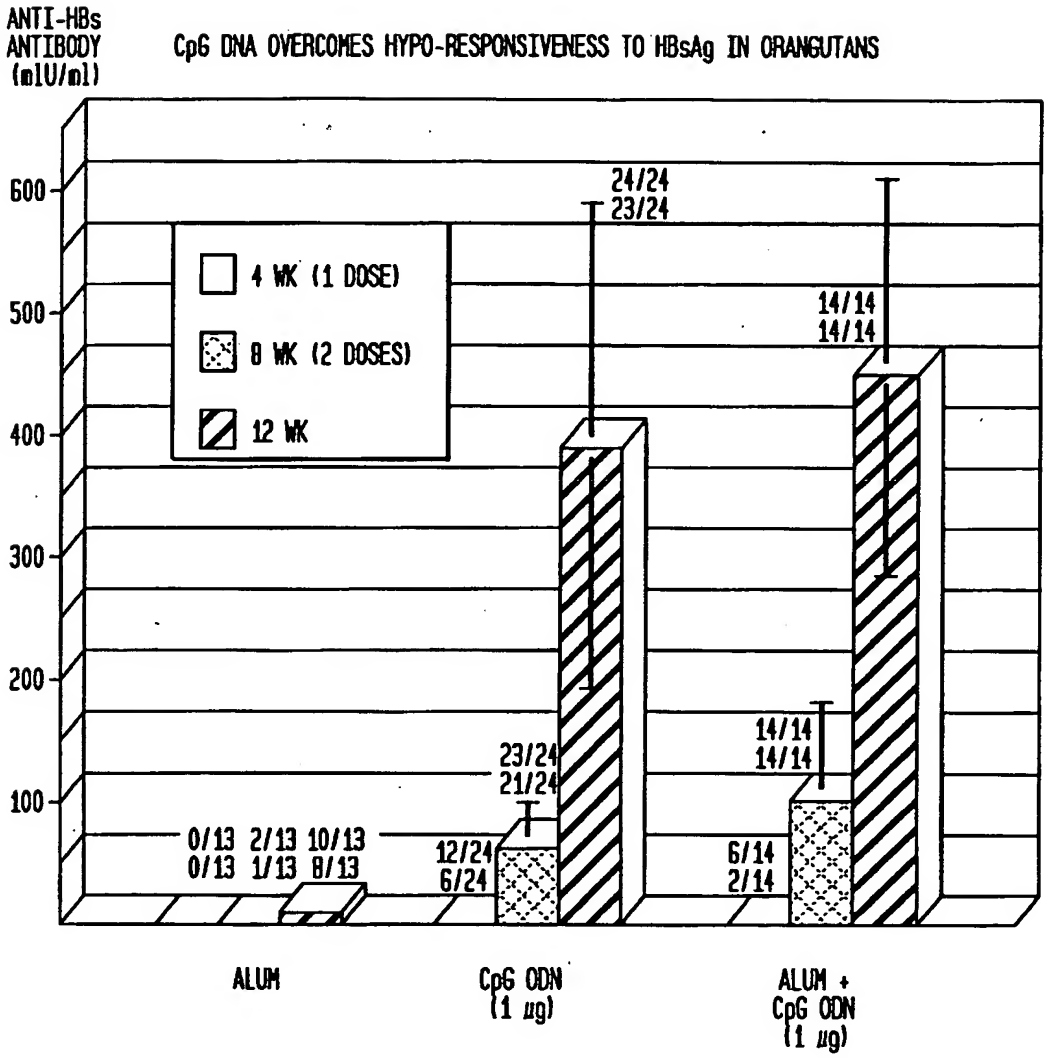

IMMUNIZATION OF CYNOMOLGUS MONKEYS AGAINST HBsAg
IMMUNOSTIMULATORY EFFECT OF CpG ODN WITH ENERGIX-B®
ENERGIX-B (10 µg HBsAg)
ALUM ONLY
CpG ODN 1968 (500 µg)
ANTI-HBs (mIU/ml)
10^6
10^5
10^4
10^3
10^2
10^1
0
0
2
4
6
8
10
12
14
TIME (WEEKS)

FIG. 12


ANTI-HBs ANTIBODY (mIU/ml)
CpG DNA OVERCOMES HYPO-RESPONSIVENESS TO HBsAg IN ORANGUTANS
600
500
400
300
200
100
4 WK (1 DOSE)
8 WK (2 DOSES)
12 WK
24/24
23/24
14/14
14/14
23/24
21/24
14/14
14/14
0/13 2/13 10/13
0/13 1/13 8/13
12/24
6/24
6/14
2/14
ALUM
CpG ODN (1 μg)
ALUM + CpG ODN (1 μg)

USE OF NUCLEIC ACIDS CONTAINING UNMETHYLATED CPG DINUCLEOTIDE AS AN ADJUVANT

RELATED APPLICATIONS

This application is a continuation in part of U.S. patent application Ser. No. 09/154,614 filed on Sep. 16, 1998, pending, which is a National Stage filing of PCT/US98/04703, filed on Mar. 10, 1998, claiming priority to U.S. Provisional Patent Application 60/040,376, filed Mar. 10, 1997, now abandoned.

FIELD OF THE INVENTION

The present invention relates generally to adjuvants, and in particular to methods and products utilizing a synergistic combination of oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) and a non-nucleic acid adjuvant.

BACKGROUND OF THE INVENTION

Bacterial DNA, but not vertebrate DNA, has direct immunostimulatory effects on peripheral blood mononuclear cells (PBMC) in vitro (Krieg et al., 1995). This lymphocyte activation is due to unmethylated CpG dinucleotides, which are present at the expected frequency in bacterial DNA (1/16), but are under-represented (CpG suppression, 1/50 to 1/60) and methylated in vertebrate DNA. Activation may also be triggered by addition of synthetic oligodeoxynucleotides (ODN) that contain an unmethylated CpG dinucleotide in a particular sequence context. It appears likely that the rapid immune activation in response to CpG DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial molecules.

CpG DNA induces proliferation of almost all (>95%) B cells and increases immunoglobulin (Ig) secretion. This B cell activation by CpG DNA is T cell independent and antigen non-specific. However, B cell activation by low concentrations of CpG DNA has strong synergy with signals delivered through the B cell antigen receptor for both B cell proliferation and Ig secretion (Krieg et al., 1995). This strong synergy between the B cell signaling pathways triggered through the B cell antigen receptor and by CpG DNA promotes antigen specific immune responses. In addition to its direct effects on B cells, CpG DNA also directly activates monocytes, macrophages, and dendritic cells to secrete a variety of cytokines, including high levels of IL-12 (Klinman et al., 1996; Halpern et al., 1996; Cowdery et al., 1996). These cytokines stimulate natural killer (NK) cells to secrete gamma-interferon (IFN-γ-) and have increased lytic activity (Klinman et al., 1996, supra; Cowdery et al., 1996, supra; Yamamoto et al., 1992; Ballas et al., 1996). Overall, CpG DNA induces a Th1 like pattern of cytokine production dominated by IL-12 and IFN-γ with little secretion of Th2 cytokines (Klinman et al., 1996).

Hepatitis B virus (HBV) poses a serious world-wide health problem. The current HBV vaccines are subunit vaccines containing particles of HBV envelope protein(s) which include several B and T cell epitopes known collectively as HBV surface antigen (HBsAg). The HBsAg particles may be purified from the plasma of chronically infected individuals or more commonly are produced as recombinant proteins. These vaccines induce antibodies against HBsAg (anti-HBs), which confer protection if present in titers of at least 10 milli-International Units per milliliter (mIU/ml) (Ellis, 1993). The current subunit vaccines which contain alum (a Th2 adjuvant), are safe and generally efficacious. They, however, fail to meet all current vaccination needs. For example, early vaccination of infants born to chronically infected mothers, as well as others in endemic areas, drastically reduces the rate of infection, but a significant proportion of these babies will still become chronically infected themselves (Lee et al., 1989; Chen et al., 1996). This could possibly be reduced if high titers of anti-HBs antibodies could be induced earlier and if there were HBV-specific CTL. In addition, there are certain individuals who fail to respond (non-responders) or do not attain protective levels of immunity (hypo-responders). Finally, there is an urgent need for an effective treatment for the estimated 350 million chronic carriers of HBV and a therapeutic vaccine could meet this need.

SUMMARY OF THE INVENTION

The present invention relates to methods and products for inducing an immune response. The invention is useful in one aspect as a method of inducing an antigen specific immune response in a subject. The method includes the steps of administering to the subject in order to induce an antigen specific immune response an antigen and a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated CpG dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount for inducing a synergistic adjuvant response. In one embodiment the subject is an infant.

The CpG oligonucleotide and the non-nucleic acid adjuvant may be administered with any or all of the administrations of antigen. For instance the combination of adjuvants may be administered with a priming dose of antigen. In another embodiment the combination of adjuvants is administered with a boost dose of antigen. In some embodiments the subject is administered a priming dose of antigen and oligonucleotide containing at least one unmethylated CpG dinucleotide before the boost dose. In yet other embodiments the subject is administered a boost dose of antigen and oligonucleotide containing at least one unmethylated CpG dinucleotide after the priming dose.

The antigen may be any type of antigen known in the art. For example, the antigen may be selected from the group consisting of peptides, polypeptides, cells, cell extracts, polysaccharides, polysaccharide conjugates, lipids, glycolipids and carbohydrates. Antigens may be given in a crude, purified or recombinant form and polypeptide/peptide antigens, including peptide mimics of polysaccharides, may also be encoded within nucleic acids. Antigens may be derived from an infectious pathogen such as a virus, bacterium, fungus or parasite, or the antigen may be a tumor antigen, or the antigen may be an allergen.

According to another aspect of the invention a method of inducing a Th1 immune response in a subject is provided. The method includes the step of administering to the subject in order to induce a Th1 immune response a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated CpG dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount for inducing a Th1 immune response. In one embodiment the combination of adjuvants is administered simultaneously. In another embodiment the combination of adjuvants is administered sequentially. In some embodiments the combination of adju-

vants is administered in an effective amount for inducing a synergistic Th1 immune response. In another aspect, the same method is performed but the subject is an infant and the Th1 response can be induced using CpG DNA alone, or CpG DNA in combination with a non-nucleic acid adjuvant at the same or different site, at the same or different time.

The invention in other aspects is a composition of a synergistic combination of adjuvants. The composition includes an effective amount for inducing a synergistic adjuvant response of a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated CpG dinucleotide and at least one non-nucleic acid adjuvant. The composition may also include at least one antigen, which may be selected from the group consisting of peptides, polypeptides, cells, cell extracts, polysaccharides, polysaccharide conjugates, lipids, glycolipids and carbohydrates. Antigens may be given in a crude, purified or recombinant form and polypeptide/peptide antigens, including peptide mimics of polysaccharides, may also be encoded within nucleic acids. Antigens may be derived from an infectious pathogen such as a virus, bacterium, fungus or parasite, or the antigen may be a tumor antigen, or the antigen may be an allergen.

According to other aspects the invention includes a method for immunizing an infant. The method involves the step of administering to an infant an antigen and an oligonucleotide containing at least one unmethylated CpG dinucleotide and at least one non-nucleic acid adjuvant in an effective amount for inducing cell mediated immunity or Th1-like responses in the infant. The method may also involve the step of administering at least one non-nucleic acid adjuvant.

The CpG oligonucleotide may be administered with any or all of the administrations of antigen. For instance the CpG oligonucleotide or the combination of adjuvants may be administered with a priming dose of antigen. In another embodiment the CpG oligonucleotide or the combination of adjuvants is administered with a boost dose of antigen. In some embodiments the subject is administered a priming dose of antigen and oligonucleotide containing at least one unmethylated CpG dinucleotide before the boost dose. In yet other embodiments the subject is administered a boost dose of antigen and oligonucleotide containing at least one unmethylated CpG dinucleotide after the priming dose.

The invention in other aspects includes a method of inducing a stronger Th1 immune response in a subject being treated with a non-nucleic acid adjuvant. The method involves the steps of administering to a subject receiving an antigen and at least one non-nucleic acid adjuvant and at least one oligonucleotide containing at least one unmethylated CpG dinucleotide in order to induce a stronger Th1 immune response than either the adjuvant or oligonucleotide produces alone.

The invention in other aspects include a method of inducing a non-antigen-specific Th1-type immune response, including Th1 cytokines such as IL-12 and IFN-γ, for temporary protection against various pathogens including viruses, bacteria, parasites and fungi. The method involves the steps of administering to a subject at least one non-nucleic acid adjuvant and at least one oligonucleotide containing at least one unmethylated CpG dinucleotide in order to induce a Th1 innate immune response. For longer term protection, these adjuvants may be administered more than once. In another embodiment, CpG DNA may be used alone at one or more of the administrations.

In each of the above described embodiments a CpG oligonucleotide is used as an adjuvant. The oligonucleotide in one embodiment contains at least one unmethylated CpG dinucleotide having a sequence including at least the following formula:

$$5'X_1X_2CGX_3X_4 3'$$

wherein C and G are unmethylated, wherein X_1X_2 and X_3X_4 are nucleotides. In one embodiment the $5'X_1X_2CGX_3\ X_4\ 3'$ sequence is a non-palindromic sequence.

The oligonucleotide may be modified. For instance, in some embodiments at least one nucleotide has a phosphate backbone modification. The phosphate backbone modification may be a phosphorothioate or phosphorodithioate modification. In some embodiments the phosphate backbone modification occurs on the 5' side of the oligonucleotide or the 3' side of the oligonucleotide.

The oligonucleotide may be any size. Preferably the oligonucleotide has 8 to 100 nucleotides. In other embodiments the oligonucleotide is 8 to 40 nucleotides in length.

In some embodiments X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA. Preferably X_1X_2 are GpA or GpT and X_3X_4 are TpT. In other preferred embodiments X_1 or X_2 or both are purines and X_3 or X_4 or both are pyrimidines or X_1X_2 are GpA and X_3 or X_4 or both are pyrimidines. In one embodiment X_2 is a T and X_3 is a pyrimidine. The oligonucleotide may be isolated or synthetic.

The invention also includes the use of a non-nucleic acid adjuvant in some aspects. The non-nucleic acid adjuvant in some embodiments is an adjuvant that creates a depo effect, an immune stimulating adjuvant, or an adjuvant that creates a depo effect and stimulates the immune system. Preferably the adjuvant that creates a depo effect is selected from the group consisting of alum (e.g., aluminum hydroxide, aluminum phosphate) emulsion based formulations including mineral oil, non-mineral oil, water-in-oil or oil-in-water emulsions, such as the Seppic ISA series of Montanide adjuvants; MF-59; and PROVAX. In some embodiments the immune stimulating adjuvant is selected from the group consiting of saponins purified from the bark of the Q. saponaria tree, such as QS21; poly[di(carboxylatophenoxy) phosphazene (PCPP) derivatives of lipopolysaccharides such as monophosphorlyl lipid (MPL), muramyl dipeptide (MDP) and threonyl muramyl dipeptide (tMDP); OM-174; and Leishmania elongation factor. In one embodiment the adjuvant that creates a depo effect and stimulates the immune system is selected from the group consiting of ISCOMS; SB-AS2; SB-AS4; non-ionic block copolymers that form micelles such as CRL 1005; and Syntex Adjuvant Formulation.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 has two graphs illustrating humoral and cytotoxic T-lymphocyte (CTL) responses in adult BALB/c mice immunized with 1 μg recombinant HBsAg protein alone, adsorbed onto alum (25 mg Al^{3+}/mg HBsAg), with 100 μg of immunostimulatory CpG ODN 1826, or with both alum and CpG ODN. Left panel: Each point represents the group mean (n=10) for titers of anti-HBs (total IgG) as determined

in triplicate by end-point dilution ELISA assay. End-point titers were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of control non-immune plasma with a cut-off value of 0.05. Rightpanel: Each point represents the mean % specific lysis at the indicated effector: target (E:T) cell ratio in a chromium release assay with HBsAg-expressing cells as targets.

FIG. 2 is a graph illustrating humoral responses in adult BALB/c mice immunized with 1 μg recombinant HBsAg protein, with or without alum, and with 0, 0.1, 1, 10, 100 or 500 μg of CpG ODN 1826 added. Each point represents the group mean (n=10) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 3 is a graph illustrating humoral responses in adult BALB/c mice immunized with 1 μg recombinant HBsAg protein, with or without alum, and with one of several different oligonucleotides (ODN, 10 μg). The ODN were made with a natural phosphodiester backbone (O), synthetic phosphorothioate backbone (S) or a chimeric of phosphodiester center regions and phosphorothioate ends (SOS). Most of the ODN contained 1–3 CpG motifs but some of the ODN were non-CpG controls (1911, 1982, 2041). Each point represents the group mean (n=5) for anti-HBs titers (total IgG) as determined by end-point dilution ELISA assay.

FIG. 4 is a graph of CTL responses in adult BALB/c mice immunized with 1 μg recombinant HBsAg protein with alum (25 mg Al^{3+}/mg HBs/Ag), with 10 μg of CpG ODN 1826, or with both alum and CpG ODN. Some animals were boosted with the same or a different formulation after 8 weeks. Each point represents the group mean (n=5) for % specific lysis of HBsAg-expressing target cell at various effector:target (E:T) cell ratios.

FIG. 5 is a graph of humoral responses in BALB/c mice immunized with HBsAg (1 μg) without adjuvant or with various adjuvants alone or in combination. The adjuvants were: alum (25 mg Al^{3+}/mg HBs/Ag), with CpG DNA (10 jig CpG ODN 1826), monophosphoryl lipid A (MPL, 50 μg) and Freund's complete adjuvant (mixed 1:1 v/v with HBsAg solution). Each point represents the group mean (n=0) for anti-HBS titers (total IgG) as determined by end-point dilution ELISA assay 4 weeks after immunization.

FIG. 6 is a bar graph depicting the amount of total IgG (end-point ELISA titer) produced at 4 weeks in BALB/c mice immunized with 1 μg of HBsAg with or without CpG and/or IFA (mineral oil mixed 1:1 v/v) or CFA (complete Freund's adjuvant mixed 1:1 v/v). The numbers above each bar indicate the IgG2a:IgG1 ratio, with a number in excess of 1 indicating a more Th1-like response.

FIG. 7 is a bar graph depicting the amount of total IgG produced at 4 weeks in BALB/c mice immunized with 1 μg of HBsAg with or without CpG and/or MPL (monophosphoryl lipid A, 50 μg) or alum. The numbers above each bar indicate the IgG2a:IgG1 ratio, with a number in excess of 1 indicating a more Th1-like response.

FIG. 8 is a graph of the percent of young BALB/c mice that seroconverted (end-point dilution titer >7100) after immunization at <1, 3, 7 or 14 days of age. Mice were immunized with 10 μg HBsAg-expressing DNA vaccine (pCMV-S), or with recombinant HBsAg (1 μg) with alum (25 mg Al3+/mg HBsAg), CpG ODN 1826 (10 μg) or both alum and CpG ODN. Each point represents the proportion of mice responding, the numbers above the bars show the number of responding over the total number immunized.

FIG. 9 has two graphs illustrating humoral and cytotoxic T-lymphocyte (CTL) responses in BALB/c mice immunized at 7 days of age with a DNA vaccine (1 μg pCMV-S), or with 1 μg recombinant HBsAg protein alone, adsorbed onto alum (25 mg Al^{3+}/mg HBsAg), with 100 μg of immunostimulatory CpG ODN 1826, or with both alum and CpG ODN. Upper panel: Each point represents the group mean of animals that seroconverted (see FIG. 8 for numbers of animals) for titers of anti-HBs (total IgG) as determined in triplicate by end-point dilution ELISA assay. End-point titers were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of control non-immune plasma with a cut-off value of 0.05. Lower panel: Each point represents the mean % specific lysis at the indicated effector: target (E:T) cell ratio in a chromium release assay with HBsAg-expressing cells as targets.

FIG. 10 is a bar graph illustrating humoral responses in neonatal BALB/c mice at 8 weeks after immunization (at 7 days of age) with 1 μg recombinant HBsAg protein with alum (25 mg Al^{3+}/mg HBsAg), with 10 μg of CpG ODN 1826, or with both alum and CpG ODN. Each point represents the group mean (see FIG. 8 for numbers of animals) for anti-HBs titers (IgG1 and IgG2a isotypes) as determined by end-point dilution ELISA assay. IgG1 antibodies indicate a Th2-biased response whereas IgG2a antibodies are indicative of a Th1-type response.

FIG. 11 is a graph of humoral responses in juvenile Cynomolgus monkeys immunized with Engerix-B vaccine (10 μg recombinant HBsAg protein with alum, SmithKline Beecham biologicals, Rixensart, BE) or with Engerix-B plus 500 μg of CpG ODN 1968. Each point represents the group mean (n=5) for anti-HBs titers in milli-International units/ml (mIU/ml). A titer of 10 mIU/ml is considered protective in humans.

FIG. 12 is a bar graph depicting titers of antibodies against HBsAg (anti-HBs) in millilnternational Units per millilitre (mIU/ml) in orangutans immunized with 10 μg HBsAg with alum (like the HBV commercial vaccine), CpG oligonucleotides (CpG ODN 2006, 1 mg) or both alum and CpG ODN. The numbers above the bars show the number of animals with seroconversion (upper numbers, >1 mIU/ml) or with seroprotection (lower numbers, >10 mIU/ml) over the total number of animals immunized. A titer of 10 mIU/ml is considered sufficient to protect humans and great apes against infection.

DETAILED DESCRIPTION OF THE INVENTION

The invention in one aspect is based on the discovery that formulations containing combinations of immunostimulatory CpG oligonucleotides and non-nucleic acid adjuvants synergistically enhance immune responses to a given antigen. Different non-nucleic acid adjuvants used in combination in the prior art have different affects on immune system activation. Some combinations of adjuvants produce an antigen-specific response that is no better than the best of the individual components and some combinations even produce lower antigen specific responses than with the individual adjuvants used alone. In Gordon et al., for instance, when humans were immunized with C terminal recombinant malaria circumsporozite antigen with alum alone or alum in combination with monophosphoryl lipid A (MPL), the subjects receiving alum alone developed higher antigen specific antibodies at several time points than subjects receiving the combination of adjuvants.

It has been discovered according to the invention that the combination of immunostimulatory CpG oligonucleotides and alum, MPL and other adjuvants results in a synergistic immune response. Compared with the recombinant hepatitis

B surface antigen (HBsAg) protein vaccine alone, addition of alum increases the level of antibodies in mice against HBsAg (anti-HBs) about 7-fold whereas addition of CpG ODN increases them 32-fold. When CpG ODN and alum are used together, a 500–1000 times higher level of anti-HBs was observed, indicating a strong synergistic response. Additionally, it was found according to the invention that immunization with HBsAg and alum resulted in a strong Th2-type response with almost all IgG being of the IgG1 isotype. CpG ODN induced a high proportion of IgG2a, indicative of a Th1-type response, even in the presence of alum. Furthermore, it was discovered according to the invention that in very young mice (7 day old), immune responses were induced by HBsAg with alum and CpG ODN but not with alum or CpG ODN alone. The antibodies produced with CpG ODN were predominantly of the IgG2a isotype, indicating a strong Th1-type response. This is remarkable considering the strong Th2 bias of the neonatal immune system and the known difficulty in inducing Th1 responses at such a young age. Th1 responses are preferable in some instances since they are associated with IgG2a antibodies that have better neutralization and opsonization capabilities than Th2-type antibodies. As well, Th1 responses are associated with cytotoxic T lymphocytes (CTL) that can attack and kill virus-infected cells. Indeed, CpG ODN, alone or in combination with alum induced good CTL activity in both adult and neonatal mice. These studies demonstrate that the addition of CpG ODN to protein or DNA vaccines in combination with other adjuvants is a valid new adjuvant approach to improve efficacy.

Thus in one aspect the invention is a method of inducing an antigen specific immune response in a subject. The method includes the step of administering to the subject in order to induce an antigen specific immune response an antigen and a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated CpG dinucleotide and at least one non-nucleic acid adjuvant, and wherein the combination of adjuvants is administered in an effective amount for inducing a synergistic adjuvant response.

The synergistic combination of adjuvants is particularly useful as a prophylactic vaccine for the treatment of a subject at risk of developing an infection with an infectious organism or a cancer in which a specific cancer antigen has been identified or an allergy where the allergen is known. The combination of adjuvants can also be given without the antigen or allergen for shorter term protection against infection, allergy or cancer, and in this case repeated doses will allow longer term protection. A "subject at risk" as used herein is a subject who has any risk of exposure to an infection causing pathogen or a cancer or an allergen or a risk of developing cancer. For instance, a subject at risk may be a subject who is planning to travel to an area where a particular type of infectious agent is found or it may be a subject who through lifestyle or medical procedures is exposed to bodily fluids which may contain infectious organisms or even any subject living in an area that an infectious organism or an allergen has been identified. Subjects at risk of developing infection also include general populations to which a medical agency recommends vaccination with a particular infectious organism antigen. If the antigen is an allergen and the subject develops allergic responses to that particular antigen and the subject is exposed to the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen.

There is a need for a prophylactic vaccine that can induce protective immunity against many infectious pathogens more quickly and with fewer doses than traditional vaccines can provide. For instance, fewer than 20% of healthy individuals attain protective levels of anti-hepatitis B (HB) antibodies (10 mIU/ml) after a single dose of subunit hepatitis B Vaccine (HBV) vaccine and only 60–70% reach this level after two doses. Thus, three doses (usually given at 0, 1 and 6 months) are required to seroconvert >90% of vaccinated individuals. The three dose regime is frequently not completed owing to poor patient compliance, and in endemic areas, protective levels may not be induced quickly enough. The methods of the invention are particularly useful as prophylactic treatments because they induce higher levels of antibodies than can be achieved with traditional vaccines and can be administered as fewer total doses.

Additionally between 5 and 10% of individuals are non-responders or hypo-responders to the subunit HBsAg vaccine. This may be MHC-restricted (Kruskall et al., 1992) and is thought to result from a failure to recognize T-helper epitopes. In certain immunocompromised individuals (e.g., kidney dialysis patients, alcoholics) the rate of non-response can approach 50%. As set forth in the Examples below, alum plus CpG ODN gave higher anti-HBs titers than alum alone in a strain of mice which has MHC-restricted hypo-responsiveness to HBsAg, thought to result in a failure to recognize T-helper epitopes. CpG ODN also overcame non-response in mice genetically incapable of providing T-help owing to an absence of class II MHC. Similar results were obtained in orangutans at risk of becoming infected with hepatitis B. It was found that orangutans are hyporesponders to the classical alum-adjuvanted vaccine with less than 10% achieving seroprotection after 2 doses, but that nearly 100% of animals responded with use of CpG oligonucleotides alone or combined with alum. The synergistic response was evident because antibody titers were much higher with CpG ODN plus alum than with CpG ODN alone or alum alone and were more than additive. These results support the proposition that CpG ODN drives the T cell independent activation of B cells. Thus in addition to providing a more effective and easier vaccination protocol the synergistic combination of adjuvants can be used to reduce the percentage of non-responders and hypo-responders.

A subject at risk of developing a cancer is one who is who has a high probability of developing cancer. These subjects include, for instance, subjects having a genetic abnormality, the presence of which has been demonstrated to have a correlative relation to a higher likelihood of developing a cancer and subjects exposed to cancer causing agents such as tobacco, asbestos, or other chemical toxins, or a subject who has previously been treated for cancer and is in apparent remission. When a subject at risk of developing a cancer is treated with an antigen specific for the type of cancer to which the subject is at risk of developing and an adjuvant and a CpG oligonucleotide the subject may be able to kill of the cancer cells as they develop. If a tumor begins to form in the subject, the subject will develop a specific immune response against the tumor antigen.

In addition to the use of the combination of adjuvants for prophylactic treatment, the invention also encompasses the use of the combination for the immunotherapeutic treatment of a subject having an infection, an allergy or a cancer. A "subject having an infection" is a subject that has been exposed to an infectious pathogen and has acute or chronic detectable levels of the pathogen in the body. The combination of adjuvants can be used with an antigen to mount an antigen specific immune response that is capable of reducing the level of or eradicating the infectious pathogen. An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body.

Many types of infectious pathogens do not have any effective treatments and chronic presence of the pathogen can result in significant damage. For instance, the HBV virus is itself non-pathogenic but with chronic infection the partially developed immune response causes inflammatory changes that eventually leads to cirrhosis and increased risk of hepatocellular carcinoma. An estimated one million people die each year from HBV-related liver disease. Persistent HBV infection of the liver results when acute infection fails to launch an appropriate immune response to clear the virus. Such chronic carriers have circulating HBsAg "e" soluble form of the HBV core antigen (HBeAg) without specific immunity. It is thought that the absence of HBV-specific T-cells, including CTL may contribute to the establishment and maintenance of the chronic carrier state. Indeed, many previously infected individuals, even years after clinical and serological recovery, have traces of HBV in their blood and HBV-specific CTL that express activation markers indicative of recent contact with antigen (Rehermann et al., 1996). These results suggest that sterilizing immunity may not occur after HBV infection and that chronic activation of HBV-specific CD4+ and CD8+ T-cells is responsible for keeping the virus under control.

There is currently no cure for the HBV chronic infection. Interferon is used currently but this cures only 10–20% of treated individuals (Niederau et al., 1996). Anti-viral drugs (e.g., lamivudine) can reduce circulating virus to undetectable levels, however these return to pretreatment levels if the drug is stopped. Each of these types of treatment is also expensive and has certain undesirable side-effects. Thus the synergistic combination of adjuvants which induces potent Th1 responses, including CTL, is useful for treating a subject having an infection such as HBV.

A "subject having an allergy" is a subject that has or is at risk of developing an allergic reaction in response to an allergen. An "allergy" refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include but are not limited to eczema, allergic rhinitis or coryza, conjunctivitis, hay fever, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions.

Currently, allergic diseases are generally treated either symptomatically with antihistimines for example or immunotherapeutically by the injection of small doses of antigen followed by subsequent increasing dosage of antigen. Symptomatic treatment offers only temporary relief. Immunotherapy is believed to induce tolerance to the allergen to prevent further allergic reactions. This approach, however, takes several years to be effective and is associated with the risk of side effects such as anaphylactic response. The methods of the invention avoid these problems.

Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by unmethylated CpG oligonucleotides are predominantly of a class called "Th1" which includes IL-12 and IFN-γ. In contrast, Th2 immune response are associated with the production of IL-4, IL-5 and IL-10. Th1responses include both cell-mediated responses (including cytotoxic T-cells) and antibodies, whereas Th2 responses are associated only with antibodies. The antibodies with a Th1 response are of isotypes (e.g. IgG2a) that have better neutralizing and opsonizing capabilities than those of Th2 isotypes (e.g. IgE that mediates allergic responses). In general, it appears that allergic diseases are mediated by Th2 type immune responses and protective immune responses by Th1 immune response although exaggerated Th1 responses may be also associated with autoimmune diseases.

Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotype switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN-γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. "Asthma" refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

Based on the ability of the CpG oligonucleotides to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose of a CpG oligonucleotide can be administered to a subject to treat or prevent an allergy.

Since Th1 responses are even more potent with CpG DNA combined with non-nucleic acid adjuvants, the combination of adjuvants of the present invention will have significant therapeutic utility in the treatment of allergic conditions such as asthma. Such combinations of adjuvants could be used alone or in combination with allergens.

A "subject having a cancer" is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. Cancers or tumors include but are not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas.

A "subject" shall mean a human or vertebrate animal including but not limited to a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, (e.g., monkey), fish (aquaculture species e.g. salmon, trout and other salmonids), rat, and mouse.

The subject is administered a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated CpG dinucleotide and at least one non-nucleic acid adjuvant. An oligonucleotide containing at least one unmethylated CpG dinucleotide is a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing a 5' cytosine followed by 3' guanosine and linked by a phosphate bond) and activates the immune system. The CpG oligonucleotides can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable in vivo, while single-stranded molecules have increased immune activity. The CpG oligonucleotides or combination of adjuvants can be used with or without antigen.

The terms "nucleic acid" and "oligonucleotide" are used interchangeably to mean multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). As used herein, the terms refer to oligoribonucleotides as well as oligodeoxyribonucleotides. The terms shall also include polynucleosides (i.e. a polynucleotide minus the phosphate) and any other organic base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g. genomic or

cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis). The entire CpG oligonucleotide can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must be unmethylated.

In one preferred embodiment the invention provides a CpG oligonucleotide represented by at least the formula:

$$5'N_1X_1CGX_2N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymine; X_2 is cytosine, adenine, or thymine; N is any nucleotide and N_1 and N_2 are nucleic acid sequences composed of from about 0–25 N's each.

In another embodiment the invention provides an isolated CpG oligonucleotide represented by at least the formula:

$$5'N_1X_1X_2CGX_3X_4N_2 3'$$

wherein at least one nucleotide separates consecutive CpGs; X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA; N is any nucleotide and N_1 and N_2 are nucleic acid sequences composed of from about 0–25 N's each. Preferably X_1X_2 are GpA or GpT and X_3X_4 are TpT. In other preferred embodiments X_1 or X_2 or both are purines and X_3 or X_4 or both are pyrimidines or X_1X_2 are GpA and X_3 or X_4 or both are pyrimidines. In a preferred embodiment N_1 and N_2 of the nucleic acid do not contain a CCGG or CGCG quadmer or more than one CCG or CGG trimer. The effect of a a CCGG or CGCG quadmer or more than one CCG or CGG trimer depends in part on the status of the oligonucleotide backbone. For instance, if the oligonucleotide has a phosphodiester backbone or a chimeric backbone the inclusion of these sequences in the oligonucleotide will only have minimal if any affect on the biological activity of the oligonucleotide. If the backbone is completely phosphorothioate or significantly phosphorothioate then the inclusion of these sequences may have more influence on the biological activity or the kinetics of the biological activity. In another preferred embodiment the CpG oligonucleotide has the sequence $5'TCN_1TX_1X_2CGX_3X_4 3'$.

Preferably the CpG oligonucleotides of the invention include X_1X_2 selected from the group consisting of GpT, GpG, GpA and ApA and X_3X_4 is selected from the group consisting of TpT, CpT and GpT. For facilitating uptake into cells, CpG containing oligonucleotides are preferably in the range of 8 to 30 bases in length. However, nucleic acids of any size greater than 8 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if sufficient immunostimulatory motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include a CCGG or CGCG quadmer or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals. Stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone modification, as discussed in more detail below are also preferred. The modification may be, for example, a phosphorothioate or phosphorodithioate modification. Preferably, the phosphate backbone modification occurs at the 5' end of the nucleic acid for example, at the first two nucleotides of the 5' end of the oligonucleotide. Further, the phosphate backbone modification may occur at the 3' end of the nucleic acid for example, at the last five nucleotides of the 3' end of the nucleic acid. Alternatively the oligonucleotide may be completely or partially modified.

Preferably the CpG oligonucleotide is in the range of between 8 and 100 and more preferably between 8 and 30 nucleotides in size. Alternatively, CpG oligonucleotides can be produced on a large scale in plasmids. These may be administered in plasmid form or alternatively they can be degraded into oligonucleotides.

The CpG oligonucleotide and immunopotentiating cytokine may be directly administered to the subject or may be administered in conjunction with a nucleic acid delivery complex. A "nucleic acid/cytokine delivery complex" shall mean a nucleic acid molecule and/or cytokine associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g. dendritic cell surfaces and/or increased cellular uptake by target cells). Examples of nucleic acid/cytokine delivery complexes include nucleic acids/cytokines associated with: a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes should be sufficiently stable in vivo to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable under appropriate conditions within the cell so that the nucleic acid/cytokine is released in a functional form.

"Palindromic sequence" shall mean an inverted repeat (i.e. a sequence such as ABCDEE'D'C'B'A' in which A and A' are bases capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double-stranded structures. In one embodiment the CpG oligonucleotide contains a palindromic sequence. A palindromic sequence used in this context refers to a palindrome in which the CpG is part of the palindrome, and preferably is the center of the palindrome. In another embodiment the CpG oligonucleotide is free of a palindrome. A CpG oligonucleotide that is free of a palindrome is one in which the CpG dinucleotide is not part of a palindrome. Such an oligonucleotide may include a palindrome in which the CpG is not part of the palindrome.

A "stabilized nucleic acid molecule" shall mean a nucleic acid molecule that is relatively resistant to in vivo degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG oligonucleotides that are tens to hundreds of kbs long are relatively resistant to in vivo degradation, particularly when in a double-stranded closed-circular form (i.e., a plamid). For shorter CpG oligonucleotides, secondary structure can stabilize and increase their effect. For example, if the 3' end of an oligonucleotide has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop structure, then the oligonucleotide becomes stabilized and therefore exhibits more activity.

Preferred stabilized oligonucleotides of the instant invention have a modified backbone. It has been demonstrated that modification of the oligonucleotide backbone provides enhanced activity of the CpG oligonucleotides when administered in vivo. CpG constructs, including at least two phosphorothioate linkages at the 5' end of the oligonucleotide in multiple phosphorothioate linkages at the 3' end, preferably 5, provides maximal activity and protected the oligonucleotide from degradation by intracellular exo- and endo-nucleases. Other modified oligonucleotides include phosphodiester modified oligonucleotide, combinations of phosphodiester and phosphorothioate oligonucleotide, methylphosphonate, methylphosphorothioate, phosphorodithioate, and combinations thereof. Each of these combinations and their particular effects on immune cells is

discussed in more detail in copending PCT Published Patent Applications claiming priority to U.S. Ser. Nos. 08/738,652 and 08/960,774, filed on Oct. 30, 1996 and Oct. 30, 1997 respectively, the entire contents of which is hereby incorporated by reference. It is believed that these modified oligonucleotides may show more stimulatory activity due to enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered intracellular localization.

Both phosphorothioate and phosphodiester oligonucleotides containing CpG motifs are active in immune cells. However, based on the concentration needed to induce CpG specific effects, the nuclease resistant phosphorothioate backbone CpG oligonucleotides are more potent (2 μg/ml for the phosphorothioate vs. a total of 90 μg/ml for phosphodiester).

Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

The nucleic acid sequences of the invention which are useful as adjuvants are those broadly described above and disclosed in PCT Published Patent Applications claiming priority to U.S. Ser. Nos. 08/738,652 and 08/960,774, filed on Oct. 30, 1996 and Oct. 30, 1997 respectively. Exemplary sequences include but are not limited to those immunostimulatory sequences shown in Table 1.

The stimulation index of a particular immunostimulatory CpG DNA can be tested in various immune cell assays. Preferably, the stimulation index of the CpG oligonucleotide with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of ^{3}H uridine in a murine B cell culture, which has been contacted with 20 μM of oligonucleotide for 20 h at 37° C. and has been pulsed with 1 μCi of ^{3}H uridine; and harvested and counted 4 h later as described in detail in copending PCT Published Patent Applications claiming priority to U.S. Ser. Nos. 08/38,652 and 08/960,774, filed on Oct. 30, 1996 and Oct. 30, 1997 respectively. For use in vivo, for example, it is important that the CpG oligonucleotide and adjuvant be capable of effectively inducing activation of Ig expressing B cells. Oligonucleotides which can accomplish this include, for example, but are not limited to those oligonucleotides described in PCT Published Patent Applications claiming priority to U.S. Ser. Nos. 08/738,652 and 08/960,774, filed on Oct. 30, 1996 and Oct. 30, 1997 respectively.

The oligonucleotide containing at least one unmethylated CpG is used in combination with a non-nucleic acid adjuvant and an antigen to activate the immune response. A "non-nucleic acid adjuvant" is any molecule or compound except for the CpG oligonucleotides described herein which can stimulate the humoral and/or cellular immune response. Non-nucleic acid adjuvants include, for instance, adjuvants that create a depo effect, immune stimulating adjuvants, and adjuvants that create a depo effect and stimulate the immune system. In infants, the oligonucleotide containing at least one unmethylated CpG is used alone or in combination with a non-nucleic acid adjuvant and an antigen to activate a cellular immune response.

An "adjuvant that creates a depo effect" as used herein is an adjuvant that causes the antigen to be slowly released in the body, thus prolonging the exposure of immune cells to the antigen. This class of adjuvants includes but is not limited to alum (e.g., aluminum hydroxide, aluminum phosphate); or emulsion-based formulations including mineral oil, non-mineral oil, water-in-oil or oil-in-water-in oil emulsion, oil-in-water emulsions such as Seppic ISA series of Montanide adjuvants (e.g., Montanide ISA 720, AirLiquide, Paris, France); MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, Calif.; and PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a micelle-forming agent; IDEC, Pharmaceuticals Corporation, San Diego, Calif.).

An "immune stimulating adjuvant" is an adjuvant that causes activation of a cell of the immune system. It may, for instance, cause an immune cell to produce and secrete cytokines. This class of adjuvants includes but is not limited to saponins purified from the bark of the Q. saponaria tree, such as QS21 (a glycolipid that elutes in the 21st peak with HPLC fractionation; Aquila Biopharmaceuticals, Inc., Worcester, Mass.); poly[di(carboxylatophenoxy) phosphazene (PCPP polymer; Virus Research Institute, USA); derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, Mont.), muramyl dipeptide (MDP; Ribi) andthreonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland); and Leishmania elongation factor (a purified Leishmania protein; Corixa Corporation, Seattle, Wash.).

"Adjuvants that create a depo effect and stimulate the immune system" are those compounds which have both of the above-identified functions. This class of adjuvants includes but is not limited to ISCOMS Immunostimulating complexes which contain mixed saponins, lipids and form virus-sized particles with pores that can hold antigen; CSL, Melbourne, Australia); SB-AS2 (SmithKline Beecham adjuvant system #2 which is an oil-in-water emulsion containing MPL and QS21: SmithKline Beecham Biologicals [SBB], Rixensart, Belgium); SB-AS4 (SmithKline Beecham adjuvant system #4 which contains alum and MPL; SBB, Belgium); non-ionic block copolymers that form micelles such as CRL 1005 (these contain a linear chain of hydrophobic polyoxpropylene flanked by chains of polyoxyethylene; Vaxcel, Inc., Norcross, Ga.); and Syntex Adjuvant Formulation (SAF, an oil-in-water emulsion containing Tween 80 and a nonionic block copolymer; Syntex Chemicals, Inc., Boulder, Colo.).

When the CpG oligonucleotide containing at least one unmethylated CpG is administered in conjunction with another adjuvant, the CpG oligonucleotide can be administered before, after, and/or simultaneously with the other adjuvant. For instance, the combination of adjuvants may be administered with a priming dose of antigen. Either or both of the adjuvants may then be administered with the boost dose. Alternatively, the combination of adjuvants may be administered with a boost dose of antigen. Either or both of the adjuvants may then be administered with the prime dose. A "prime dose" is the first dose of antigen administered to the subject. In the case of a subject that has an infection the prime dose may be the initial exposure of the subject to the infectious microbe and thus the combination of adjuvants is administered to the subject with the boost dose. A "boost dose" is a second or third, etc, dose of antigen administered to a subject that has already been exposed to the antigen. In some cases the prime dose administered with the combination of adjuvants is so effective that a boost dose is not

required to protect a subject at risk of infection from being infected. In cases where the combination of adjuvants is given without antigen, with repeated administrations, CpG oligonucleotides or one of the components in the combination may be given alone for one or more of the administrations.

The CpG oligonucleotide containing at least one unmethylated CpG can have an additional efficacy (e.g., antisense) in addition to its ability to enhance antigen-specific immune responses.

An "antigen" as used herein is a molecule capable of provoking an immune response. Antigens include but are not limited to peptides, polypeptides, cells, cell extracts, polysaccharides, polysaccharide conjugates, lipids, glycolipids and carbohydrates. Antigens may be given in a crude, purified or recombinant form and polypeptide/peptide antigens, including peptide mimics of polysaccharides, may also be encoded within nucleic. The term antigen broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens include but are not limited to cancer antigens, microbial antigens, and allergens.

A "cancer antigen" as used herein is a compound, such as a peptide, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, et al., 1994, *Cancer Research*, 54:1055, by partially purifying the antigens, by recombinant technology, or by de novo synthesis of known antigens. Cancer antigens include antigens that are recombinately an immunogenic portion of or a whole tumor or cancer. Such antigens can be isolated or prepared recombinatly or by any other means known in the art.

A "microbial antigen" as used herein is an antigen of a microorganism and includes but is not limited to infectious virus, infectious bacteria, infectious parasites and infectious fingi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives thereof and also synthetic compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar to a natural microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Most such antigens are used routinely in the art and are well known to those of ordinary skill in the art. Another example is a peptide mimic of a polysaccharide antigen.

Examples of infectious virus that have been found in humans include but are not limited to: Retroviridae (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g. strains that cause gastroenteritis); Togaviridae (e.g. equine encephalitis viruses, rubella viruses); Flaviridae (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); Coronoviridae (e.g. coronaviruses); Rhabdoviradae (e.g. vesicular stomatitis viruses, rabies viruses); Coronaviridae (e.g. coronaviruses); Rhabdoviridae (e.g. vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g. ebola viruses); Paramyxoviridae (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bungaviridae (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g. reoviruses, orbiviurses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvovirida (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to Pasteurella species, Staphylococci species, and Streptococcus species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, Pseudomonas species, and Salmonella species. Specific examples of infectious bacteria include but are not limited to: Helicobacterpyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacteria sps (e.g. M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), Streptococcus (viridans group), *Streptococcusfaecalis, Streptococcus bovis*, Streptococcus (*anaerobic* sps.), *Streptococcus pneumoniae*, pathogenic Campylobacter sp., Enterococcus sp., *Haemophilus infuenzae, Bacillus antracis*, corynebacterium diphtheriae, corynebacterium sp., Erysipelothrix rhusiopathiae, Clostridium perfringers, Clostridium tetani, Enterobacter aerogenes, *Klebsiella pneumoniae, Pasturella multocida*, Bacteroides sp., *Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira, Rickettsia*, and *Actinomyces israelli*.

Examples of infectious fingi include: *Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans*. Examples of infectious parasites include Plasmodium such as *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale*, and *Plasmodium vivax*. Other infectious organisms (i.e. protists) include Toxoplasma gondii.

Other medically relevant microorganisms have been descried extensively in the literature, e.g., see C. G. A Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

Although many of the microbial antigens described above relate to human disorders, the invention is also useful for treating other nonhuman vertebrates. Nonhuman vertebrates are also capable of developing infections which can be prevented or treated with the synergistic combination of adjuvants disclosed herein. For instance, in addition to the treatment of infectious human diseases, the methods of the invention are useful for treating infections of animals.

As used herein, the term "treat","treated", or "treating" when used with respect to an infectious disease refers to a prophylactic treatment which increases the resistance of a subject (a subject at risk of infection) to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen, as well as a treatment after the subject (a subject who has been

infected) has become infected in order to fight the infection, e.g., reduce or eliminate the infection or prevent it from becoming worse. Many vaccines for the treatment of non-human vertebrates are disclosed in Bennett, K. *Compendium of Veterinary Products*, 3rd ed. North American Compendiums, Inc., 1995. As discussed above, antigens include infectious microbes such as virus, bacteria, parasites and fungi and fragments thereof, derived from natural sources or synthetically. Infectious virus of both human and non-human vertebrates, include retroviruses, RNA viruses and DNA viruses. This group of retroviruses includes both simple retroviruses and complex retroviruses. The simple retroviruses include the subgroups of B-type retroviruses, C-type retroviruses and D-type retroviruses. An example of a B-type retrovirus is mouse mammary tumor virus (MMTV). The C-type retroviruses include subgroups C-type group A (including Rous sarcoma virus (RSV), avian leukemia virus (ALV), and avian myeloblastosis virus (AMV)) and C-type group B (including murine leukemia virus (MLV), feline leukemia virus (FeLV), murine sarcoma virus (MSV), gibbon ape leukemia virus (GALV), spleen necrosis virus (SNV), reticuloendotheliosis virus (RV) and simian sarcoma virus (SSV)). The D-type retroviruses include Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1). The complex retroviruses include the subgroups of lentiviruses, T-cell leukemia viruses and the foamy viruses. Lentiviruses include HIV-1, but also include HIV-2, SIV, Visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The T-cell leukemia viruses include HTLV-1, HTLV-II, simian T-cell leukemia virus (STLV), and bovine leukemia virus (BLV). The foamy viruses include human foamy virus (HFV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Examples of other RNA viruses that are antigens in vertebrate animals include, but are not limited to, the following: members of the family Reoviridae, including the genus Orthoreovirus (multiple serotypes of both mammalian and avian retroviruses), the genus Orbivirus (Bluetongue virus, Eugenangee virus, Kemerovo virus, African horse sickness virus, and Colorado Tick Fever virus), the genus Rotavirus (human rotavirus, Nebraska calf diarrhea virus, murine rotavirus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family Picornaviridae, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A virus, Simian enteroviruses, Murine encephalomyelitis (ME) viruses, Poliovirus muris, Bovine enteroviruses, Porcine enteroviruses , the genus Cardiovirus (Encephalomyocarditis virus (EMC), Mengovirus), the genus Rhinovirus (Human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus Apthovirus (Foot and Mouth disease (FMDV); the family Calciviridae, including Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family Togaviridae, including the genus Alphavirus (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavirius (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyvirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus of mice); forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavirius (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyvirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus of mice); the family Rhabdoviridae, including the genus Vesiculovirus (VSV), Chandipura virus, Flanders-Hart Park virus), the genus Lyssavirus (Rabies virus), fish Rhabdoviruses, and two probable Rhabdoviruses (Marburg virus and Ebola virus); the family Arenaviridae, including Lymphocytic choriomeningitis virus (LCM), Tacaribe virus complex, and Lassa virus; the family Coronoaviridae, including Infectious Bronchitis Virus (IBV), Mouse Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis (Feline coronavirus).

Illustrative DNA viruses that are antigens in vertebrate animals include, but are not limited to: the family Poxviridae, including the genus Orthopoxvirus (Variola major, Variola minor, Monkey pox Vaccinia, Cowpox, Buffalopox, Rabbitpox, Ectromelia), the genus Leporipoxvirus (Myxoma, Fibroma), the genus Avipoxvirus (Fowlpox, other avian poxvirus), the genus Capripoxvirus (sheeppox, goatpox), the genus Suipoxvirus (Swinepox), the genus

Parapoxvirus (contagious postular dermatitis virus, pseudocowpox, bovine papular stomatitis virus); the family Iridoviridae (African swine fever virus, Frog viruses 2 and 3, Lymphocystis virus of fish); the family Herpesviridae, including the alpha-Herpesviruses (Herpes Simplex Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3, pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis virus, feline rhinotracheitis virus, infectious laryngotracheitis virus) the Beta-herpesvirises (Human cytomegalovirus and cytomegaloviruses of swine, monkeys and rodents); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes saimiri, Herpesvirus ateles, Herpesvirus sylvilagus, guinea pig herpes virus, Lucke tumor virus); the family Adenoviridae, including the genus Mastadenovirus (Human subgroups A,B,C,D,E and ungrouped; simian adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of cattle, pigs, sheep, frogs and many other species, the genus Aviadenovirus (Avian adenoviruses); and non-cultivatable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RKV), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotrophic papilloma virus); the family Parvoviridae including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc). Finally, DNA viruses may include viruses which do not fit into the above families such as Kuru and Creutzfeldt-Jacob disease viruses and chronic infectious neuropathic agents (CHINA virus).

Each of the foregoing lists is illustrative, and is not intended to be limiting.

In addition to the use of the combination of CpG oligonucleotides and non-nucleic acid adjuvants to induce an antigen specific immune response in humans, the methods of the preferred embodiments are particularly well suited for treatment of birds such as hens, chickens, turkeys, ducks, geese, quail, and pheasant. Birds are prime targets for many types of infections.

Hatching birds are exposed to pathogenic microorganisms shortly after birth. Although these birds are initially protected against pathogens by maternal derived antibodies, this protection is only temporary, and the bird's own immature immune system must begin to protect the bird against the pathogens. It is often desirable to prevent infection in young birds when they are most susceptible. It is also desirable to prevent against infection in older birds, especially when the birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the CpG oligonucleotide and the non-nucleic acid adjuvant of the invention to birds to enhance an antigen-specific immune response when antigen is present. The CpG oligonucleotide and the non-nucleic acid adjuvant of the invention could also be administered to birds without antigen to protect against infection of a wide variety of pathogens.

An example of a common infection in chickens is chicken infectious anemia virus (CIAV). CIAV was first isolated in Japan in 1979 during an investigation of a Marek's disease vaccination break (Yuasa et al., 1979, Avian Dis. 23:366–385). Since that time, CIAV has been detected in commercial poultry in all major poultry producing countries (van Bulow et al., 1991, pp.690–699) in Diseases of Poultry, 9th edition, Iowa State University Press).

CIAV infection results in a clinical disease, characterized by anemia, hemorrhage and immunosuppression, in young susceptible chickens. Atrophy of the thymus and of the bone marrow and consistent lesions of CIAV-infected chickens are also characteristic of CIAV infection. Lymphocyte depletion in the thymus, and occasionally in the bursa of Fabricius, results in immunosuppression and increased susceptibility to secondary viral, bacterial, or fungal infections which then complicate the course of the disease. The immunosuppression may cause aggravated disease after infection with one or more of Marek's disease virus (MDV), infectious bursal disease virus, reticuloendotheliosis virus, adenovirus, or reovirus. It has been reported that pathogenesis of MDV is enhanced by CIAV (DeBoer et al., 1989, p. 28 In Proceedings of the 38th Western Poultry Diseases Conference, Tempe, Ariz.). Further, it has been reported that CIAV aggravates the signs of infectious bursal disease (Rosenberger et al., 1989, Avian Dis. 33:707–713). Chickens develop an age resistance to experimentally induced disease due to CAA. This is essentially complete by the age of 2 weeks, but older birds are still susceptible to infection (Yuasa, N. et al., 1979 supra; Yuasa, N. et al., Arian Diseases 24, 202–209, 1980). However, if chickens are dually infected with CAA and an immunosuppressive agent (IBDV, MDV etc.) age resistance against the disease is delayed (Yuasa, N. et al., 1979 and 1980 supra; Bulow von V. et al., J. Veterinary Medicine 33, 93–116, 1986). Characteristics of CIAV that may potentiate disease transmission include high resistance to environmental inactivation and some common disinfectants. The economic impact of CIAV infection on the poultry industry is clear from the fact that 10% to 30% of infected birds in disease outbreaks die.

Vaccination of birds, like other vertebrate animals can be performed at any age. Normally, vaccinations are performed at up to 12 weeks of age for a live microorganism and between 14–18 weeks for an inactivated microorganism or other type of vaccine. For in ovo vaccination, vaccination can be performed in the last quarter of embryo development. The vaccine may be administered subcutaneously, by spray, orally, intraocularly, intratracheally, nasally, in ovo or by other methods described herein. Thus, the CpG oligonucleotide and non-nucleic acid adjuvant of the invention can be administered to birds and other non-human vertebrates using routine vaccination schedules and the antigen is administered after an appropriate time period as described herein.

Cattle and livestock are also susceptible to infection. Disease which affect these animals can produce severe economic losses, especially amongst cattle. The methods of the invention can be used to protect against infection in livestock, such as cows, horses, pigs, sheep, and goats. The CpG oligonucleotide and the non-nucleic acid adjuvant of the invention could also be administered with antigen for antigen-specific protection of long duration or without antigen for short term protection against a wide variety of diseases, including shipping fever.

Cows can be infected by bovine viruses. Bovine viral diarrhea virus (BVDV) is a small enveloped positive-stranded RNA virus and is classified, along with hog cholera virus (HOCV) and sheep border disease virus (BDV), in the pestivirus genus. Although, Pestiviruses were previously classified in the Togaviridae family, some studies have suggested their reclassification within the Flaviviridae family along with the flavivirus and hepatitis C virus (HCV) groups (Francki, et al., 1991).

BVDV, which is an important pathogen of cattle can be distinguished, based on cell culture analysis, into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes. The

NCP biotype is more widespread although both biotypes can be found in cattle. If a pregnant cow becomes infected with an NCP strain, the cow can give birth to a persistently infected and specifically immunotolerant calf that will spread virus during its lifetime. The persistently infected cattle can succumb to mucosal disease and both biotypes can then be isolated from the animal. Clinical Manifestations can include abortion, teratogenesis, and respiratory problems, mucosal disease and mild diarrhea. In addition, severe thrombocytopenia, associated with herd epidemics, that may result in the death of the animal has been described and strains associated with this disease seem more virulent than the classical BVDVs.

Equine herpesviruses (EHV) comprise a group of antigenically distinct biological agents which cause a variety of infections in horses ranging from subclinical to fatal disease. These include Equine herpesvirus-1 (EHV-1), a ubiquitous pathogen in horses. EHV-1 is associated with epidemics of abortion, respiratory tract disease, and central nervous system disorders. Primary infection of upper respiratory tract of young horses results in a febrile illness which lasts for 8 to 10 days. Immunologically experienced mares may be reinfected via the respiratory tract without disease becoming apparent, so that abortion usually occurs without warning. The neurological syndrome is associated with respiratory disease or abortion and can affect animals of either sex at any age, leading to incoordination, weakness and posterior paralysis (Telford, E. A. R. et al., Virology 189, 304–316, 1992). Other EHV's include EHV-2, or equine cytomegalovirus, EHV-3, equine coital exanthema virus, and EHV-4, previously classified as EHV-1 subtype 2.

Sheep and goats can be infected by a variety of dangerous microorganisms including visna-maedi.

Primates such as monkeys, apes and macaques can be infected by simian immunodeficiency virus. Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in macaques (Stott et al. (1990) Lancet 36:1538–1541; Desrosiers et al. PNAS USA (1989) 86:6353–6357; Murphey-Corb et al. (1989) Science 246:1293–1297; and Carlson et al. (1990) AIDS Res. Human Retroviruses 6:1239–1246). A recombinant HIV gp120 vaccine has been reported to afford protection in chimpanzees (Berman et al. (1990) Nature 345:622–625).

Cats, both domestic and wild, are susceptible to infection with a variety of microorganisms. For instance, feline infectious peritonitis is a disease which occurs in both domestic and wild cats, such as lions, leopards, cheetahs, and jaguars. When it is desirable to prevent infection with this and other types of pathogenic organisms in cats, the methods of the invention can be used to vaccinate cats to prevent them against infection.

Domestic cats may become infected with several retroviruses, including but not limited to feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type C oncornavirus (RD-114), and feline syncytia-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms, including lymphoreticular and myeloid neoplasms, anemias, immune mediated disorders, and an immunodeficiency syndrome which is similar to human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has been more particularly associated with immunosuppressive properties.

The discovery of feline T-lymphotropic lentivirus (also referred to as feline immunodeficiency) was first reported in Pedersen et al. (1987) Science 235:790–793. Characteristics of FIV have been reported in Yamamoto et al. (1988) Leukemia, December Supplement 2:204S–215S; Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246–1258; and Ackley et al. (1990) J. Virol. 64:5652–5655. Cloning and sequence analysis of FIV have been reported in Olmsted et al. (1989) Proc. Natl. Acad. Sci. USA 86:2448–2452 and 86:4355–4360.

Feline infectious peritonitis (FIP) is a sporadic disease occurring unpredictably in domestic and wild Felidae. While FIP is primarily a disease of domestic cats, it has been diagnosed in lions, mountain lions, leopards, cheetahs, and the jaguar. Smaller wild cats that have been afflicted with FIP include the lynx and caracal, sand cat, and pallas cat. In domestic cats, the disease occurs predominantly in young animals, although cats of all ages are susceptible. A peak incidence occurs between 6 and 12 months of age. A decline in incidence is noted from 5 to 13 years of age, followed by an increased incidence in cats 14 to 15 years old.

Viral, bacterial and parasitic diseases in fin-fish, shellfish or other aquatic life forms pose a serious problem for the aquaculture industry. Owing to the high density of animals in the hatchery tanks or enclosed marine farming areas, infectious diseases may eradicate a large proportion of the stock in, for example, a fin-fish, shellfish, or other aquatic life forms facility. Prevention of disease is a more desired remedy to these threats to fish than intervention once the disease is in progress. Vaccination of fish is the only preventative method which may offer long-term protection through immunity. Fish are currently protected against a variety of bacterial infections with whole killed vaccines with oli adjuvants, but there is only one licensed vaccine for fish against a viral disease. Nucleic acid based vaccinations are described in U.S. Pat. No. 5,780,448 issued to Davis and these have been shown to be protective against at least two different viral diseases.

The fish immune system has many features similar to the mammalian immune system, such as the presence of B cells, T cells, lymphokines, complement, and immunoglobulins. Fish have lymphocyte subclasses with roles that appear similar in many respects to those of the B and T cells of mammals. Vaccines can be administered orally or by immersion or injection.

Aquaculture species include but are not limited to fin-fish, shellfish, and other aquatic animals. Fin-fish include all vertebrate fish, which may be bony or cartilaginous fish, such as, for example, salmonids, carp, catfish, yellowtail, seabream, and seabass. Salmonids are a family of fin-fish which include trout (including rainbow trout), salmon, and Arctic char. Examples of shellfish include, but are not limited to, clams, lobster, shrimp, crab, and oysters. Other cultured aquatic animals include, but are not limited to eels, squid, and octopi.

Polypeptides of viral aquaculture pathogens include but are not limited to glycoprotein (G) or nucleoprotein (N) of viral hemorrhagic septicemia virus (VHSV); G or N proteins of infectious hematopoietic necrosis virus (IHNV); VP1, VP2, VP3 or N structural proteins of infectious pancreatic necrosis virus (IPNV); G protein of spring viremia of carp (SVC); and a membrane-associated protein, tegumin or capsid protein or glycoprotein of channel catfish virus (CCV).

Polypeptides of bacterial pathogens include but are not limited to an iron-regulated outer membrane protein, (IROMP), an outer membrane protein (OMP), and an A-protein of Aeromonis salmonicida which causes furunculosis, p57 protein of Renibacterium salmoninarum which causes bacterial kidney disease (BKD), major surface

associated antigen (msa), a surface expressed cytotoxin (mpr), a surface expressed hemolysin (ish), and a flagellar antigen of Yersiniosis; an extracellular protein (ECP), an iron-regulated outer membrane protein (IROMP), and a structural protein of Pasteurellosis; an OMP and a flagellar protein of Vibrosis anguillarum and V. ordalii; a flagellar protein, an OMP protein,aroA, and purA of Edwardsiellosis ictaluri and E. tarda; and surface antigen of Ichthyophthirius; and a structural and regulatory protein of Cytophaga columnari; and a structural and regulatory protein of Rickettsia.

Polypeptides of a parasitic pathogen include but are not limited to the surface antigens of Ichthyophthirius.

An "allergen" refers to a substance (antigen) that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g. penicillin). Examples of natural, animal and plant allergens include but are not limited to proteins specific to the following genuses: Canine (*Canis familiaris*); Dermatophagoides (e.g. *Dermatophagoides farinae*); Felis (*Felis domesticus*); Ambrosia (*Ambrosia artemiisfolia*; Lolium (e.g. *Lolium perenne* or *Lolium multiflorum*); Cryptomeria (*Cryptomeria japonica*); Alternaria (*Alternaria alternata*); Alder; Alnus (*Alnus gultinoasa*); Betula (*Betula verrucosa*); Quercus (*Quercus alba*); Olea (Olea europa); Artemisia (*Artemisia vulgaris*); Plantago (e.g. *Plantago lanceolata*); Parietaria (e.g. *Parietaria officinalis* or *Parietaria judaica*); Blattella (e.g. *Blattella germanica*); Apis (e.g. *Apis multiflorum*); Cupressus (e.g. *Cupressus sempervirens, Cupressus arizonica* and *Cupressus macrocarpa*); Juniperus (e.g. *Juniperus sabinoides, Juniperus virginiana, Juniperus communis* and *Juniperus ashei*); Thuya (e.g. *Thuya orientalis*); Chamaecyparis (e.g. *Chamaecyparis obtusa*); Periplaneta (e.g. *Periplaneta americana*); Agropyron (e.g. *Agropyron repens*); Secale (e.g. *Secale cereale*); Triticum (e.g. *Triticum aestivum*); Dactylis (e.g. *Dactylis glomerata*); Festuca (e.g. *Festuca elatior*); Poa (e.g. *Poa pratensis* or *Poa compressa*); Avena (e.g. *Avena sativa*); Holcus (e.g. *Holcus lanatus*); Anthoxanthum (e.g. *Anthoxanthum odoratum*); Arrhenatherum (e.g. *PArrhenatherum elatius*); Agrostis (e.g. *Agrostis alba*); Phleum (e.g. *Phleum pratense*); Phalaris (e.g. *Phalaris arundinacea*); Paspalum (e.g. *Paspalum notatum*); Sorghum (e.g. *Sorghum halepensis*); and Bromus (e.g. *Bromus inermis*).

In some aspects of the invention the antigen is a polypeptide. Minor modifications of the primary amino acid sequences of polypeptide antigens may also result in a polypeptide which has substantially equivalent antigenic activity as compared to the unmodified counterpart polypeptide. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as antigenicity still exists. The polypeptide may be, for example, a viral polypeptide. One non-limiting example of an antigen useful according to the invention is the hepatitis B surface antigen. Experiments using this antigen are described in the Examples below.

The term "substantially purified" as used herein refers to a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify viral or bacterial polypeptides using standard techniques for protein purification. The substantially pure polypeptide will often yield a single major band on a non-reducing polyacrylamide gel. In the case of partially glycosylated polypeptides or those that have several start codons, there may be several bands on a non-reducing polyacrylamide gel, but these will form a distinctive pattern for that polypeptide. The purity of the viral or bacterial polypeptide can also be determined by amino-terminal amino acid sequence analysis.

The invention also utilizes polynucleotides encoding the antigenic polypeptides. It is envisioned that the antigen may be delivered to the subject in a nucleic acid molecule which encodes for the antigen such that the antigen must be expressed in vivo. The nucleic acid encoding the antigen is operatively linked to a gene expression sequence which directs the expression of the antigen nucleic acid within a eukaryotic cell. The "gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the antigen nucleic acid to which it is operatively linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, β-actin promoter, muscle creatine kinase promoter, human elongation factor promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the simian virus (e.g., SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), rous sarcoma virus, cytomegalovirus (CMV), Rous sarcoma virus (RSV), hepatitis B virus (HBV), the long terminal repeats (LTR) of Moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined antigen nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

The antigen nucleic acid is operatively linked to the gene expression sequence. As used herein, the antigen nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the expression or transcription and/or translation of the antigen coding sequence under the influence or control of the gene expression sequence. Two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the antigen sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the antigen sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be

operably linked to an antigen nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that antigen nucleic acid sequence such that the resulting transcript is translated into the desired protein or polypeptide.

The antigen nucleic acid sequence may encode a protein, polypeptide, peptide, or peptide mimic of a polysaccbaride. It may also encode more than one antigenic component as a fusion construct. More than one antigen-encoding sequence may be included in the same plasmid vector and these may be linked to the same or different gene expression sequences.

The antigen nucleic acid of the invention may be delivered to the immune system alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antigen nucleic acid to the cells of the immune system and preferably APCs so that the antigen can be expressed and presented on the surface of an APC. Preferably, the vector transports the nucleic acid to the immune cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. The vector optionally includes the above-described gene expression sequence to enhance expression of the antigen nucleic acid in APCs. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antigen nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual," W.H. Freeman C.O., New York (1990) and Murry, E. J. Ed. "Methods in Molecular Biology," vol. 7, Humana Press, Inc., Cliffton, N.J. (1991).

Preferred virus for certain applications are the adeno-virus and adeno-associated virus which are double-stranded DNA viruses that have already been approved for human use in gene therapy and immunotherapy trials. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well-known to those of skill in the art. See e.g., Sanbrook et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well-known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids such as those used for DNA vaccines may be delivered by a variety of parenteral, mucosal and topical routes. For example the plasmid DNA can be injected by intramuscular, intradermal, subcutaneous or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

It has recently been discovered that gene carrying plasmids can be delivered to the immune system using bacteria. Modified forms of bacteria such as Salmonella can be transfected with the plasmid and used as delivery vehicles. The bacterial delivery vehicles can be administered to a host subject orally or by other administration means. The bacteria deliver the plasmid to immune cells, e.g. dendritic cells, probably by passing through the gut barrier. High levels of immune protection have been established using this methodology.

In other aspects the invention includes a method for immunizing an infant by administering to an infant an antigen and an oligonucleotide containing at least one unmethylated CpG dinucleotide in an effective amount for inducing cell mediated immunity in the infant. In some embodiments the infant is also administered at least one non-nucleic acid adjuvant, as described above. Cell mediated immunity, as used herein, refers to an immune response which involves an antigen specific T cell reaction. The presence of cell mediated immunity can be determined directly by the induction of Th1 cytokines (e.g., IFN-γ, IL-12) and antigen-specific cytotoxic T-cell lymphocytes (CTL). The presence of cell mediated immunity is also indicated indirectly by the isotype of antigen-specific antibodies that are induced (e.g. IgG2a>>IgG1 in mice). Thus,

if Th1 cytokines or CTL or TH2-like antibodies are induced, cell mediated immunity is induced according to the invention. As discussed above, Th1 cytokines include but are not limited to IL-12 and IFN-γ.

Neonates (newborn) and infants (which include humans three months of age and referred to hereinafter as infants) born in HBV endemic areas require particularly rapid induction of strong HBV-specific immunity owing to the high rate of chronicity resulting from infection at a young age. Without immunoprophylaxis, 70–90% of infants born to mothers positive for both HBsAg and the "e" antigen (HBeAg) become infected and almost all of these become chronic carriers (Stevens et al., 1987). Even when vaccinated with a four dose regime of the HBV subunit vaccine commencing on the day of birth, 20% of such infants became chronically infected and this was reduced to only 15% if they were also given HBV-specific immunoglobulin (Chen et al. 1996) HBV chronicity results in 10–15% of individuals infected as adolescents or adults, but 90–95% for those infected (either vertically or horizontally) as infants. CpG oligonucleotides may be used, according to the invention, to reduce this further owing to a more rapid appearance and higher titers of anti-HBs antibodies and the induction of HBV-specific CTL, which could help clear virus from the liver of babies infected in utero, and which likely account for most of the failures with infant vaccination.

The invention further provides a method of modulating the level of a cytokine. The term "modulate" envisions the suppression of expression of a particular cytokine when lower levels are desired, or augmentation of the expression of a particular cytokine when higher levels are desired. Modulation of a particular cytokine can occur locally or systemically. CpG oligonucleotides can directly activate macrophages and dendritic cells to secrete cytokines. No direct activation of proliferation or cytokine secretion by highly purified T cells has been found, although they are induced to secrete cytokines by cytokines secreted from macrophages and may be costimulated through the T cell Receptor. Cytokine profiles determine T cell regulatory and effector functions in immune responses. In general, Th1-type cytokines are induced, thus the immunostimulatory nucleic acids promote a Th1 type antigen-specific immune response including cytotoxic T-cells.

Cytokines also play a role in directing the T cell response. Helper ($CD4^+$) T cells orchestrate the immune response of mammals through production of soluble factors that act on other immune system cells, including B and other T cells. Most mature $CD4^+$ T helper cells express one of two cytokine profiles: Th1 or Th2. Th1 cells secrete IL-2, IL-3, IFN-γ, GM-CSF and high levels of TNF-α. Th2 cells express IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF and low levels of TNF-α. The Th1 subset promotes both cell-mediated immunity, and humoral immunity that is characterized by immunoglobulin class switching to IgG_{2a} in mice. Th1 responses may also be associated with delayed-type hypersensitivity and autoimmune disease. The Th2 subset induces primarily humoral immunity and induce class switching to IgG_1, and IgE. The antibody isotypes associated with Th1 responses generally have good neutralizing and opsonizing capabilities whereas those associated with Th2 responses are associated more with allergic responses.

Several factors have been shown to influence commitment to Th1 or Th2 profiles. The best characterized regulators are cytokines. IL-12 and IFN-γ are positive Th1 and negative Th2 regulators. IL-12 promotes IFN-γ production, and IFN-γ provides positive feedback for IL-12. IL-4 and IL-10 appear to be required for the establishment of the Th2 cytokine profile and to down-regulate Th1 cytokine production; the effects of IL4 are in some cases dominant over those of IL-12. IL-13 was shown to inhibit expression of inflammatory cytokines, including IL-12 and TNF-α by LPS-induced monocytes, in a way similar to IL-4. The IL-12 p40 homodimer binds to the IL-12 receptor and may antagonizes IL-12 biological activity; thus it blocks the pro-Th1 effects of IL-12 in some animals.

In other aspects the invention includes a method of inducing a Th1 immune response in a subject by administering to the subject a combination of adjuvants in an effective amount for inducing a Th1 immune response. The combination of adjuvants includes at least one oligonucleotide containing at least one umnethylated CpG dinucleotide and at least one non-nucleic acid adjuvant. It was not previously known that when CpG was combined with a non-nucleic acid adjuvant, as described above, that the combination would produce an immune response with a Th1 profile to an extent that the individual adjuvants could not produce alone. Preferably the extent of the Th profile produced by the combination of adjuvants is synergistic. Another aspect of the invention is to induce a Th response by using CPG with a non-nucleic acid adjuvant that by itself induces a Th2 response.

As described above a Th2 profile is characterized by production of IL-4 and IL-10. Non-nucleic acid adjuvants that induce Th2 or weak Th1 responses include but are not limited to alum, saponins, oil-in-water and other emulsion formulations and SB-As4. Adjuvants that induce Th1 responses include but are not limited to MPL, MDP, ISCOMS, IL-12, IFN-γ, and SB-AS2. When the CpG oligonucleotide is administered with a non-nucleic acid adjuvant the combination of adjuvants causes a commitment to a Th1 profile, that neither the adjuvant nor the CpG oligonucleotide is capable of producing on its own. Furthermore, if the non-nucleic acid adjuvant on its own induces a Th2 response, the addition of CpG oligonucleotide can overcome this Th2 bias and induce a Th1 response that may be even more Th1-like than with CpG alone.

The combination of adjuvants may be administered simultaneously or sequentially. When the adjuvants are administered simultaneously they can be administered in the same or separate formulations, and in the latter case at the same or separate sites, but are administered at the same time. The adjuvants are administered sequentially, when the administration of the at least two adjuvants is temporally separated. The separation in time between the administration of the two adjuvants may be a matter of minutes or it may be longer. The separation in time is less than 14 days, and more preferably less than 7 days, and most preferably less than 1 day. The separation in time may also be with one adjuvant at prime and one at boost, or one at prime and the combination at boost, or the combination at prime and one at boost.

For use in the instant invention, the nucleic acids can be synthesized de novo using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Beaucage, S. L., and Caruthers, M. H., *Tet. Let.* 22:1859, 1981); nucleoside H-phosphonate method (Garegg et al., *Tet. Let.* 27:4051–4054, 1986; Froehler et al., *Nucl. Acid. Res.* 14:5399–5407, 1986, ; Garegg et al., *Tet. Let.* 27:4055–4058, 1986, Gaffney et al., *Tet. Let.* 29:2619–2622, 1988). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, CpG dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor laboratory Press, New York, 1989) which after being

administered to a subject are degraded into oligonucleotides. Such plasmids may also encode other genes to be expressed such as an antigen-encoding gene in the case of a DNA vaccine. Oligonucleotides can be prepared from existing nucleic acid sequences (e.g., genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

For use in vivo, nucleic acids are preferably relatively resistant to degradation (e.g., via endo-and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid has at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and alkyl-phosphonates can be made, e.g., as described in U.S. Pat. No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A., *Chem. Rev.* 90:544, 1990; Goodchild, J., *Bioconjugate Chem.* 1:165, 1990).

For administration in vivo, nucleic acids may be associated with a molecule that results in higher affinity binding to target cell (e.g., B-cell, monocytic cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells to form a "nucleic acid delivery complex." Nucleic acids can be ionically or covalently associated with appropriate molecules using techniques which are well known in the art. A variety of coupling or cross-linking agents can be used, e.g., protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Nucleic acids can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

Nucleic acids containing an appropriate unmethylated CpG can be effective in any mammal, preferably a human. Different nucleic acids containing an unmethylated CpG can cause optimal immune stimulation depending on the mammalian species. Thus an oligonucleotide causing optimal stimulation in humans may not cause optimal stimulation in a mouse and vice versa. One of skill in the art can identify the optimal oligonucleotides useful for a particular mammalian species of interest using routine assays described herein and/or known in the art.

The CpG ODN of the invention stimulate cytokine production (e.g., IL-6, IL-1 2, IFN-γ, TNF-α and GM-CSF) and B-cell proliferation in PBMC's taken from a subject such as a human. Specific, but nonlimiting examples of such sequences include those presented in Table 1 below:

TABLE 1

sequences	
GCTAGACGTTAGCGT;	(SEQ ID NO: 1)
GCTAGATGTTAGCGT;	(SEQ ID NO: 2)
GCTAGACGTTAGCGT;	(SEQ ID NO: 3)
GCTAGACGTTAGCGT;	(SEQ ID NO: 4)
GCATGACGTTGAGCT;	(SEQ ID NO: 5)
ATGGAAGGTCCAGCGTTCTC;	(SEQ ID NO: 6)
ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 7)
ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 8)
ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 9)
ATGGAAGGTCCAACGTTCTC;	(SEQ ID NO: 10)

TABLE 1-continued

sequences	
GAGAACGCTGGACCTTCCAT;	(SEQ ID NO: 11)
GAGAACGCTCGACCTTCCAT;	(SEQ ID NO: 12)
GAGAACGCTCGACCTTCGAT;	(SEQ ID NO: 13)
GAGAACGCTGGACCTTCCAT;	(SEQ ID NO: 14)
GAGAACGATGGACCTTCCAT;	(SEQ ID NO: 15)
GAGAACGCTCCAGCACTGAT;	(SEQ ID NO: 16)
TCCATGTCGGTCCTGATGCT;	(SEQ ID NO: 17)
TCCATGTCGGTCCTGATGCT;	(SEQ ID NO: 18)
TCCATGACGTTCCTGATGCT;	(SEQ ID NO: 19)
TCCATGTCGGTCCTGCTGAT;	(SEQ ID NO: 20)
TCAACGTT;	(SEQ ID NO: 21)
TCAGCGCT;	(SEQ ID NO: 22)
TCATCGAT;	(SEQ ID NO: 23)
TCTTCGAA;	(SEQ ID NO: 24)
CAACGTT;	(SEQ ID NO: 25)
CCAACGTT;	(SEQ ID NO: 26)
AACGTTCT;	(SEQ ID NO: 27)
TCAACGTC;	(SEQ ID NO: 28)
ATGGACTCTCCAGCGTTCTC;	(SEQ ID NO: 29)
ATGGAAGGTCCAACGTTCTC;	(SEQ ID NO: 30)
ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 31)
ATGGAGGCTCCATCGTTCTC;	(SEQ ID NO: 32)
ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 33)
ATCGACTCTCGAGCGTTCTC;	(SEQ ID NO: 34)
TCCATGTCGGTCCTGATGCT;	(SEQ ID NO: 35)
TCCATGCCGGTCCTGATGCT;	(SEQ ID NO: 36)
TCCATGGCGGTCCTGATGCT;	(SEQ ID NO: 37)
TCCATGACGGTCCTGATGCT;	(SEQ ID NO: 38)
TCCATGTCGATCCTGATGCT;	(SEQ ID NO: 39)
TCCATGTCGCTCCTGATGCT;	(SEQ ID NO: 40)
TCCATGTCGTCCCTGATGCT;	(SEQ ID NO: 41)
TCCATGACGTGCCTGATGCT;	(SEQ ID NO: 42)
TCCATAACGTTCCTGATGCT;	(SEQ ID NO: 43)
TCCATGACGTCCCTGATGCT;	(SEQ ID NO: 44)
TCCATCACGTGCCTGATGCT;	(SEQ ID NO: 45)
GGGGTCAACGTTGACGGGG;	(SEQ ID NO: 46)
GGGGTCAGTCGTGACGGGG;	(SEQ ID NO: 47)
GCTAGACGTTAGTGT;	(SEQ ID NO: 48)
TCCATGTCGTTCCTGATGCT;	(SEQ ID NO: 49)
ACCATGGACGATCTGTTTCCCCTC;	(SEQ ID NO: 50)
TCTCCCAGCGTGCGCCAT;	(SEQ ID NO: 51)
ACCATGGACGAACTGTTTCCCCTC;	(SEQ ID NO: 52)
ACCATGGACGAGCTGTTTCCCCTC;	(SEQ ID NO: 53)
ACCATGGACGACCTGTTTCCCCTC;	(SEQ ID NO: 54)
ACCATGGACGTACTGTTTCCCCTC;	(SEQ ID NO: 55)
ACCATGGACGGTCTGTTTCCCCTC;	(SEQ ID NO: 56)
ACCATGGACGTTCTGTTTCCCCTC;	(SEQ ID NO: 57)
CACGTTGAGGGGCAT;	(SEQ ID NO: 58)
TCAGCGTGCGCC;	(SEQ ID NO: 59)
ATGACGTTCCTGACGTT;	(SEQ ID NO: 60)
TCTCCCAGCGGGCGCAT,	(SEQ ID NO: 61)
TCCATGTCGTTCCTGTCGTT;	(SEQ ID NO: 62)
TCCATAGCGTTCCTAGCGTT;	(SEQ ID NO: 63)
TCGTCGCTGTCTCCCCTTCTT;	(SEQ ID NO: 64)
TCCTGACGTTCCTGACGTT;	(SEQ ID NO: 65)
TCCTGTCGTTCCTGTCGTT;	(SEQ ID NO: 66)
TCCATGTCGTTTTTGTCGTT;	(SEQ ID NO: 67)
TCCTGTCGTTCCTTGTCGTT;	(SEQ ID NO: 68)
TCCTTGTCGTTCCTGTCGTT;	(SEQ ID NO: 69)
TCCTGTCGTTTTTTGTCGTT;	(SEQ ID NO: 70)
TCGTCGCTGTCTGCCCTTCTT;	(SEQ ID NO: 71)
TCGTCGCTGTTGTCGTTTCTT;	(SEQ ID NO: 72)
TCCATGCGTGCGTGCGTTTT;	(SEQ ID NO: 73)
TCCATGCGTTGCGTTGCGTT;	(SEQ ID NO: 74)
TCCACGACGTTTTCGACGTT;	(SEQ ID NO: 75)
TCGTCGTTGTCGTTGTCGTT;	(SEQ ID NO: 76)
TCGTCGTTTTGTCGTTTTGTCGTT;	(SEQ ID NO: 77)
TCGTCGTTGTCGTTTTGTCGTT;	(SEQ ID NO: 78)
GCGTGCGTTGTCGTTGTCGTT;	(SEQ ID NO: 79)
TGTCGTTTGTCGTTTGTCGTT;	(SEQ ID NO: 80)
TGTCGTTGTCGTTGTCGTTGTCGTT;	(SEQ ID NO: 81)
TGTCGTTGTCGTTGTCGTT;	(SEQ ID NO: 82)
TCGTCGTCGTCGTT;	(SEQ ID NO: 83)
TGTCGTTGTCGTT;	(SEQ ID NO: 84)
TCCATAGCGTTCCTAGCGTT;	(SEQ ID NO: 85)
TCCATGACGTTCCTGACGTT;	(SEQ ID NO: 86)
GTCGTT;	(SEQ ID NO: 87)

TABLE 1-continued

sequences	
TGTCGTT;	(SEQ ID NO: 88)
AGCTATGACGTTCCAAGG;	(SEQ ID NO: 89)
TCCATGACGTTCCTGACGTT;	(SEQ ID NO: 90)
ATCGACTCTCGAACGTTCTC;	(SEQ ID NO: 91)
TCCATGTCGGTCCTGACGCA;	(SEQ ID NO: 92)
TCTTCGAT;	(SEQ ID NO: 93)
ATAGGAGGTCCAACGTTCTC;	(SEQ ID NO: 94)
GTCGTT	(SEQ ID NO: 95)
GTCGTC	(SEQ ID NO: 96)
TGTCGTT	(SEQ ID NO: 97)
TGTCGCT	(SEQ ID NO: 98)

Preferred CpG ODN can effect at least about 500 pg/ml of TNF-α, 15 pg/ml IFN-γ, 70 pg/ml of GM-CSF 275 pg/ml of IL-6, 200 pg/ml IL-12, depending on the therapeutic indication. These cytokines can be measured by assays well known in the art. The oligonucleotides listed above or other preferred CpG ODN can effect at least about 10%, more preferably at least about 15% and most preferably at least about 20% YAC-1 cell specific lysis or at least about 30%, more preferably at least about 35%, and most preferably at least about 40% 2C11 cell specific lysis, in assays well known in the art.

The term "effective amount" of a CpG oligonucleotide refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of an oligonucleotide containing at least one unmethylated CpG and a non-nucleic acid adjuvant for treating an infectious disorder is that amount necessary to cause the development of an antigen specific immune response upon exposure to the microbe, thus causing a reduction in the amount of microbe within the subject and preferably to the eradication of the microbe. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular CpG oligonucleotide being administered (e.g. the number of unmethylated CpG motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular adjuvant and antigen without necessitating undue experimentation.

The formulations of the invention are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.

For use in therapy, an effective amount of the adjuvant combination can be administered to a subject by any mode allowing the oligonucleotide to be taken up by the appropriate target cells. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Preferred routes of administration include but are not limited to oral, transdermal (e.g. via a patch), parenteral injection (subcutaneous, intradermal, intravenous, parenteral, intraperitoneal, intrathecal, etc.), or mucosal intranasal, intratracheal, inhalation, and intrarectal, intravaginal etc). An injection may be in a bolus or a continuous infusion.

For example the pharmaceutical compositions according to the invention are often administered by intramuscular or intradermal injection, or other parenteral means, or by biolistic "gene-gun" application to the epidermis. They may also be administered by intranasal application, inhalation, topically, intravenously, orally, or as implants, and even rectal or vaginal use is possible. Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for injection or inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, *Science* 249:1527–1533, 1990, which is incorporated herein by reference.

The pharmaceutical compositions are preferably prepared and administered in dose units. Liquid dose units are vials or ampoules for injection or other parenteral administration. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, manner of administration, purpose of the immunization (i.e., prophylactic or therapeutic), nature and severity of the disorder, age and body weight of the patient, different doses may be necessary. The administration of a given dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units. Multiple administration of doses at specific intervals of weeks or months apart is usual for boosting the antigen-specific responses.

The adjuvants and antigens may be administered per se (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1–2% w/v); citric acid and a salt (1–3% w/v); boric acid and a salt (0.5–2.5% w/v); and phosphoric acid and a salt (0.8–2% w/v). Suitable preservatives include benzalkonium chloride (0.003–0.03% w/v); chlorobutanol (0.3–0.9% w/v); parabens (0.01–0.25% w/v) and thimerosal (0.004–0.02% w/v).

The pharmaceutical compositions of the invention contain an effective amount of a combination of adjuvants and antigens optionally included in a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" means one or more compatible solid or liquid filler, dilutants or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being comingled with the compounds of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

Compositions suitable for parenteral administration conveniently comprise sterile aqueous preparations, which can be isotonic with the blood of the recipient. Among the acceptable vehicles and solvents are water, Ringer's solution, phosphate buffered saline and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed mineral or non-mineral oil may be employed including synthetic mono-ordi-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for subcutaneous, intramuscular, intraperitoneal, intravenous, etc. administrations may be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa.

The adjuvants or antigens useful in the invention may be delivered in mixtures of more than two adjuvants or antigens. A mixture may consist of several adjuvants in addition to the synergistic combination of adjuvants or several antigens.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular adjuvants or antigen selected, the age and general health status of the subject, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are discussed above.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compounds into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compounds, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Pat. No. 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di-and triglycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent of the invention is contained in a form within amatrix such as those described in U.S. Pat. Nos. 4,452,775, 4,675,189, and 5,736, 152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Pat. Nos. 3,854,480, 5,133,974 and 5,407, 686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

The use of CpG ODN as an adjuvant alone or in combination with other adjuvants was evaluated. The hepatitis B virus surface antigen (HBsAg) given as a recombinant protein or expressed in vivo from a DNA vaccine was used as an exemplary model system in the Examples set forth below.

MATERIALS AND METHODS

Animals

Experiments on adult mice were carried out using female BALB/c mice (Charles River, Montreal, QC) at 6–8 weeks of age.

Newborn mice were obtained through breeding male and female BALB/c mice (Charles River) in the Loeb animal facility (Loeb Health Research Institute, The Ottawa Hospital, Ottawa, ON). Pregnant females were monitored daily to ensure accurate recording of the date of birth. Both male and female neonates were used for immunization.

Cynomolgus monkeys (1.5–3 kg) were housed at the Primate Research Center, Bogor, Indonesia.

Orantutans (5–20kg) were housed at Wanariset Station for the Orangutan Reintroduction Program of the Indonesian government, Balikpapan, Kalimantan.

HBsAg Subunit Vaccination of Mice

The subunit vaccine consisted of HBsAg (ay subtype) which had been produced as a recombinant protein in yeast cells (Medix Biotech #ABH0905). This was diluted in saline for use without adjuvant. HBsAg was also formulated with alum and/or CpG ODN as adjuvant. HBsAg protein was mixed with aluminum hydroxide (Alhydrogel 85, [Al_2O_3], Superfos Biosector, Vedbaek, Denmark) in the same ratio of 25 mg A^{3+} per mg protein as used in the commercial vaccines (i.e., 2.5 12% Al_2O_3 per μg HBsAg). The protein and alum were mixed with a vortex and then left on ice for at least 30 minutes prior to use to allow the protein to adsorb onto the Al_2O_3. This solution was mixed again immediately prior to injection by drawing up into the syringe 3–5 times.

For groups treated with CpG ODN, an appropriate volume of synthetic oligodeoxynucleotide (ODN # 1826) of the sequence TCCATGACGTTCCTGACGTT (SEQ ID NO. 86) synthesized with a phosphorothioate backbone (Oligos Etc. & Oligo Therapeutics, Wilsonville, Oreg.) was added alone or with alum to HBsAg on the day of injection. Adult mice received a single intramuscular (IM) injection into the left tibialis anterior (TA) muscle of 1 or 2 ug HBsAg, without or with adjuvant (alum and/or CpG ODN), in 50 1 vehicle. When CpG DNA was added, each animal received a total of 1, 10, 100 or 500 μg ODN. Newborn mice were immunized within 24 hours of birth or 7 days after birth by bilateral injection of a total of 1 μg HBsAg into the posterior thigh muscles (2x101 α@ 0.05 mg/ml). All injections were carried out with a 0.3 ml insulin syringe which has a fused 29G needle (Becton Dickenson, Franklin Lakes, N.J.). For injection of adults, the needle was fitted with a collar of polyethylene (PE) tubing to limit penetration of the needle to about 3 mm. All intramuscular injections were carried out through the skin (shaved for adults) and under general anesthesia (Halothane, Halocarbon Laboratories, River Edge, N.J.).

HBsAg Subunit Vaccination of Monkeys

Monkeys were immunized by IM injection into the anterior thigh muscle of Engerix-B® (SmithKline Beecham Biologicals, Rixensart, BE) which comprises HBsAg (ay subtype, 20 μg/ml) adsorbed to alum (25 mg Al3+/mg HbsAg). Each monkey received an injection of 0.5 ml containing 10 μg HbsAg. For some monkeys, 500 μg CpG ODN 1968 (TCGTCGCTGTTGTCGTTTCTT) (SEQ ID NO 72) was added to the vaccine formulation.

HBsAg Subunit Vaccination of Orangutans

Orangutans were immunized by IM injection into the anterior thigh muscle of HBsAg *ay subtype, 20 μg/ml) combined with alum (25 mg Al3+/mg HBsAg), combined with CpG. CpG ODN 2006 (TCGTCGTGTCGTGTCGTT) (SEQ ID NO 77) was added to the vaccine formulation. Each orangutan received an injection of 1.0 ml containing 20 μg HBsAg with alum (500 μg), CpG oligonucleotide (1 mg) or both adjuvants.

Experimental Groups

Comparison of CpG ODN and Non-nucleic Acid Adjuvants with HBsAg Subunit Vaccine

Twelve groups of adult BALB/c mice (n=10) were injected with 1 μg HBsAg (i) alone, (ii) mixed with alum, (iii, iv, v, vi, vii) mixed with 0.1, 1, 10, 100 or 500 μg CpG ODN, or (viii, ix, x, xi, xii) mixed with both alum and 0.1, 1, 10, 100 or 500 μg CpG ODN. These mice were bled at 1, 2, 4 and 8 weeks after immunization and the plasma was assayed for anti-HBs. At the end of the study the mice were killed and their spleens removed for assay of CTL activity.

Other groups of mice (n=5) were immunized with HBsAg (1 μg) alone, with alum (25 μg A13+), with one of several different CpG and non-CpG control oligonucleotides of different backbones (10 μg), or with both alum and an oligonucleotide.

Other groups of mice (n=5) were immunized as above (except only the 10 μg dose of CpG ODN was used) and boosted with the identical or a different formulation at 8 weeks, then spleens were removed 2 weeks later for evaluation of CTL activity.

Other groups of mice were immunized with HBsAg (1 μg) and one of the following non-nucleic acid adjuvants alone or in combination with CpG ODN (10 μg): monophosphoryl lipid A (MPL, 50 μg, Ribi); Freund's Complete Adjuvant (CFA; 1:1 v/v); Freund's Incomplete Adjuvant (IFA; 1:1 v/v).

Immunization of Neonates with Subunit or DNA Vaccine

Groups of newborn and young BALB/c mice (n=10) aged <24 hours, 3, 7 or 14 days were injected with (i, ii, iii) a total of 1 μg HBsAg with alum, with CpG ODN 1826 (10 μg) or with both alum and CpG ODN, or with (iv) an HBsAg-expressing DNA vaccine (1-μg pCMV-S). Plasma was obtained at 4, 8, 12 and 16 weeks for assay of anti-HBs as total IgG and IgG subtypes (IgG1 and IgG2a). At the end of the study the mice were killed and their spleens removed for assay of CTL activity.

Immunization of Cynomolgus Monkeys with HBsAg and Alum or Alum+CpG ODN

Two groups of juvenile Cynomolgus monkeys (n=5) were immunized at 0 and 10 weeks with 0.5 ml Engerix-B (HBsAg at 20 mg/ml adsorbed to alum, 25 mg Al3+/mg HBsAg) to which had been added saline (0.1 ml) or CpG ODN 2006 (500 μg in 0.1 ml, SEQ #77). Monkeys were bled at 2, 8, 10, 12 and 14 weeks and plasma was evaluated for anti-HBs titers (mIU/ml).

Immunization of Orangutans with HBsAg and Alum or CpG ODN or Alum+CpG ODN

Three groups ofjuvenile orangutans were immunized IM at 0 and 4 weeks with 1 ml of vaccine containing HBsAg (10 μg) plus (i) alum (25 mg Al3+/mg HBsAg)(n=13), (ii) CpG ODN 2006 (SEQ# 77) (m=24) or (iii) alum plus CpG ODN (n=14). Animals were bled at 4.8 and 12 weeks and plasma was evaluated for anti-HBs titers (mIU/ml).

Evaluation of Humoral Response to HBsAg

Mice: Heparinized blood was collected by retrobulbar puncture of lightly anaesthetized mice as described elsewhere (Michel et al., 1995). Plasma was recovered by centrifugation (7 min @ 13,000 rpm). Antibodies specific to HBsAg in plasma were detected and quantified by end-point dilution ELISA assay (in triplicate) on individual samples. Ten-fold serial dilutions of plasma were first added to 96-well microtiter plates with a solid phase consisting of plasma-derived HBsAg particles (100 L/well of HBsAg ay subtype at 1 g/ml, coated overnight at RT) and incubated for 1 hr at 37C. The bound antibodies were then detected by incubation for 1 hr at 37C with HRP-conjugated goat anti-mouse IgG, IgM, IgG1 or IgG2a (1:4000 in PBS-Tween, 10% FCS; 100 l/well, Southern Biotechnology Inc., Birmingham, Ala.), followed by incubation with OPD solution (100 l/well, Sigma, St. Louis, Mo.) for 30 minutes at RT in the dark. The reaction was stopped by the addition of sulfuric acid (50 1 of 4N H_2SO_4). End-point titers were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of non-immune plasma with a cut-off value of 0.05. Anti-HBs titers were expressed as group means of individual animal values, which were themselves the average of triplicate assays.

Primates: Monkeys and orangutans were bled by antecubital venous puncture into heparinized tubes and plasma was recovered by centrifugation (7 min @ 13,000 rpm). Plasma was then evaluated for anti-HBs titers using a commercial kit (Monolisa anti-HBs, Pasteur-Sanofi) and expressed in milli-International Units per milliliter (mIU/ml) by comparison with standards defined by the World Health Organization. A titer of 10 mIU/ml is considered sufficient to confer protection to humans and great apes against infection by HBV.

Evaluation of Cytotoxic T cell Response to HBsAg in Mice

Cytotoxic T-lymphocyte (CTL) activity was determined using splenocytes taken from mice 4 or 8 weeks post-prime or post-boost. In brief, single cell suspensions were prepared and suspended in tissue culture medium (RPMI 1640, 10% FBS, Life Technologies, Grand Island, N.Y., supplemented with 5×10^{-5} M β-mercaptoethanol and penicillinstreptomycin solution, 1000 U/ml, 1 mg/ml fmal concentrations respectively, Sigma, and 3% El-4 supernatant as a source of IL-2). Splenocytes (3×10^7) were co-cultured for 5 days (37° C., 5% CO2) with 1×10^6 syngenic HBsAg-expressing stimulator cells (P815S) or control target cells (P815) in round bottom 96-well culture plates (37° C., 5% CO2, 4 hr). Supernatant (100 μl) was removed for radiation (gamma) counting. Spontaneous release was determined by incubating target cells without effector cells and total release by addition of 100 μl 2 N HCl to the target cells. The percent lysis was calculated as [(experimental release—spontaneous release)/(total release—spontaneous release)]×100. The percent specific lysis was calculated as % lysis with P815S-%

lysis with P815 cells, CTL activity for responding mice [% specific lysis >10] were expressed as means±SEM of individual animal values, which were themselves the average of triplicate assays.

Example 1

Synergy of CpG ODN with Alum as Adjuvant for HBV Subunit Vaccine in Mice

A. Strength and Kinetics of Humoral Response

Immunization of BALB/c mice with HBsAg alone elicited only low titers of anti-HBs (<100) by 4 weeks. These titers were about 10-fold higher with the addition of alum as adjuvant, 60-fold higher with CpG ODN and more than 500-fold higher with both alum and CpG ODN. At later time points, the highest peak titers were with HBsAg/alum/CpG, the second highest with HBsAg/CpG, then HBsAg/alum (FIG. 1).

Similar synergistic results for antibody responses were obtained when immunization against HBsAg was carried out in neonatal and very young mice, in which the immune system is immature. In mice immunized at 3 days of age, where the immune system is even less mature than a newborn human, 10% and 0% of mice seroconverted with alum and CpG ODN alone respectively, but 75% serocoinverted when CpG ODN and alum were used together. In 7 day old mice, which have an immune system similar in maturity to that of a newborn human, seroconversion for alum, CpG or the combination was 11%, 22% and 1 00% respectively (FIG. 8). Furthermore, in these 7 day old mice, antibody titers were up to 80-fold higher with the combined adjuvants than with either adjuvant alone (FIG. 9).

When used alone or combined with alum, there is a dose-response for CpG ODN with the best results being obtained with an intermediate dose (10 μg) and no further or only relatively small gains with higher doses (up to 500 μg) when used alone or combined with alum respectively (FIG. 2).

When a large panel of ODN is compared for adjuvant activity it can be seen that CPG ODN with a nuclease-resistant phosphorothioate backbone have the best adjuvant effects (FIG. 3). There was very little or no adjuvant activity of non-CpG control ODN with a phosphorothioate backbone, or of CpG ODN with a chimeric or phosphodiester backbone. However, for those phosphorothioate CpG ODN that did not have adjuvant effect, all exhibited a synergistic effect with alum. In general, antibody titers with combined alum and CpG ODN were 10 to 100-fold higher than with CpG ODN and/or 100 to 1000-fold higher than with alum alone (FIG. 3).

B. Strength of Cytotoxic T-lymphocyte Response

CTL were very weak with HBsAg and no adjuvant, and were completely lost with the addition of alum. CTL were augmented equally with both CpG ODN as with combined alum and CpG ODN (FIG. 1). A synergy for CTL responses could be seen with prime-boost strategies, in that priming with CpG ODN and boosting with alum gave better CTL than priming and boosting with CpG alone (FIG. 4) (Note: use of alum alone completely abrogates the CTL response).

A synergistic action of CpG ODN and alum on CTL was very evident with immunization of young (7 day old) mice. In this case, neither alum nor CpG ODN used alone induced significant levels of HBsAg-specific CTL, but when used together there wre very strong CTL were observed (FIG. 9).

Thus, CpG ODN is superior to alum for both humoral and cell-mediated responses, when each is used alone as adjuvant with the HBsAg subunit vaccine in mice. When used together, there is a synergy of action such that antibody and CTL activity are stronger than when either adjuvant is used alone. These results indicate that CpG ODN could be used to replace alum in vaccine formulations, which could be desirable to avoid associated side-effects due to local irritation in the muscle, or for certain live-attenuated or multivalent vaccines where it is not possible to use alum because chemical interactions interfere with the efficacy of the vaccine. This should not occur with CpG ODN. Of even greater interest is the strong synergistic response when CpG ODN and alum are used together as adjuvants. This could allow better immune responses with lower or fewer doses of antigen. There is a fairly flat dose response to CpG ODN whether or not alum is present, indicating that a wide range of CpG ODN could be useful to adjuvant vaccines in humans.

Example 2

Synergy of CpG ODN with Other Non-nucleic Acid Adjuvants for HBV Subunit Vaccine in Mice.

As discussed above, CpG ODN alone gave 8-fold higher antibody titers than alum, the only adjuvant currently licensed for human use. It also produces superior results to monphosphoryl lipid A (MPL, Ribi Pharmaceuticals, Middleton, Wis.), a new adjuvant that is currently in human clinical trials even when administered in a dose of five times less than that of MPL. There was, as discussed above, a strong synergy with CpG ODN and alum, but in contrast no such synergy was seen with MPL and alum. Owing to the strong synergistic effect of alum and CpG ODN, this combination of adjuvants is even better than Freund's complete adjuvant (FCA) for inducing antibodies in mice (FIG. 5) Freund's, which is considered the gold standard adjuvant for animal models is much too toxic to use in humans.

The synergy seen with CpG ODN and alum, was also seen with CpG ODN combined with other adjuvants. When used alone, CpG ODN and Freund's incomplete adjuvant (FIA, a type of mineral oil) induced similar antibody titers, but when used together the anti-HBs titers were more than 50-fold higher than with either adjuvant alone. Indeed, the combination of CpG ODN and FIA was even better than FCA (FIG. 6).

Similarly, CpG ODN and MPL alone gave equally high antibody titers, but when used together the titers were about 4-times higher than with either adjuvant alone (FIG. 7). While the synergistic response with CpG and MPL was not as marked with respect to overall antibody titers, it was very pronounced with respect to the Th1-bias of these antibodies (see below).

Example 3

Dominance and Synergy of CpG ODN with Alum for Induction of a Th1-type immune response including CTL

Immunization with either HBsAg alone or with alum induces a predominantly Th2-type humoral response with virtually no IgG2a antibodies, which are induced in response to Th1 -type cytokines such as IL-12 and IFN-γ. Rather, almost all (>99%) antibodies were of the IgG1 isotype IgG2a:IgG1=0.01. CpG ODN induces significantly more IgG2a antibodies, such that they made up at least 50% of the total IgG (IgG (IgG2a:IgG1=1.4). The combination of alum and CpG ODN induce an equally strong Th1 response as CpG ODN alone (IgG2a:IgG1=1.0), despite the extremely strong Th2-bias of alum (FIG. 5). Similarly CTL responses

with CpG ODN plus alum were as strong as those with CpG ODN alone, despite the fact that the Th2-bias of alum resulted in a complete loss of CTL when alum was used alone (FIG. 1).

The strong Th1 bias with CpG is even more evident in neonatal and young mice, which are known to naturally have a strong Th2-bias to their immune system. In this case, neither alum nor CpG ODN on their own induced detectable IgG2a, indicating a very poor or absent Th1 response. Remarkably, when used together, CpG ODN and alum induced high levels of Ig G2a antibodies, which were now the predominant form of IgG (FIG. 10). Similarly, neither CpG ODN or alum induced significant levels of CTL in young mice, yet when used together there was a strong CTL response, that was even stronger than obtained with a DNA vaccine (FIG. 9).

The strength of the Th1 influence of CpG ODN is seen not only by its ability to dominate over the Th2 effect of alum when they are co-administered, but also to induce Th1 responses in animals previously primed for a Th2 response with alum. Immunization with HBsAg using alum as an adjuvant completely abrogates the CTL response owing to the strong Th2 bias of alum (FIGS. 1 and 4). However, in mice using alum at prime and CpG at boost, good CTL were induced, indicating the possibility of CpG to overcome a previously established Th2 response (FIG. 4).

Aluminum hydroxide (alum) is currently the only adjuvant approved for human use. An important disadvantage of alum is that it induces a Th2- rather than a Th1-type immune response, and this may interfere with induction of CTL. Indeed, in mice immunized with recombinant HBsAg, the addition of alum selectively blocked activation of $CD8^+$ CTL (Schirmbeck et al., 1994). Although not essential for protective immunity against HBV, CTL may nevertheless play an important role. For example, a lack of HBV-specific CTL is thought to contribute to the chronic carrier state. In contrast, one of the primary advantages of CpG DNA over alum as an adjuvant is the Th1-bias of the responses and thus the possibility to induce CTL. A striking finding from the present study is that CpG can completely counteract the Th2-bias of alum when the two adjuvants are delivered together, and in the case of immunization in early life, the combination can even give a more Th1 response than CpG ODN alone. This could allow one to capitalize on the strong synergistic action of the two adjuvants on the humoral response while still allowing CTL in adults, and to induce a stronger Th1 response in infants.

The use of alum has been linked to Th2-type diseases. The much higher prevalence of asthma (another Th2-type disease) in more highly developed nations may be linked to the high hygiene level and rapid treatment of childhood infections (Cookson and Moffatt, 1997). Early exposure to bacterial DNA (and immunostimulatory CpG motifs) pushes the immune system away from Th2- and towards a Th1-type response and this may account for the lower incidence of asthma in less developed countries, where there is a much higher frequency of upper respiratory infections during childhood. Addition of CpG ODN as adjuvant to all pediatric vaccines could re-establish a Th1-type response thereby reducing the incidence of asthma.

Example 4

Synergy of CpG ODN with Other Adjuvants for Induction of a Th1-type Immune Responses

The synergistic effect of CpG ODN on Th1 responses was also seen using other adjuvants. IFA on its own induces a very strong Th2-type response with virtually no IgG2a antibodies (IgG2a:IgG1=0.002) and CpG ODN on its own induces a moderate Th1 response (IgG2a:IgG1=1.4), but together the response was very strongly Th1 (IgG2a:IgG1=24.0). It is notable that this is even more Th1 than the response induced by CFA (ratio=0.5) (FIG. 6).

Similarly, CpG and MPL on their own are moderately Th1 (IgG2a:IgG1 ratios at 4 weeks are 1.4 and 1.9 respectively), but together are very strongly Th1 with a large predominance of IgG2a antibodies (ratio=83.3)(FIG.7).

Example 5

CpG ODN as Synergistic Adjuvant in Cynomolgus Monkeys

CpG ODN, in combination with alum, also acts as a potent adjuvant to augment anti-HBs responses in Cynomolgus monkeys. Compared to responses obtained with the commercial HBV vaccine that contains alum, monkeys immunized with the commercial vaccine plus CpG ODN attained titers 50-times higher after prime and 10-times higher after boost (FIG. 14).

Example 6

CpG ODN as Synergistic Adjuvant to HBsAg in Hyporesponder Orangutans

The orangutans, a great ape very closely related to man, can be naturally infected with HBV in a manner similar to humans. Unfortunately, orangutans are hyporesponsive to the commercial HBV vaccine that contains alum as an adjuvant. Compared to humans, where 13% and 56% of vaccinated individuals seroconvert by 4 weeks after first and second doses respectively (Yano and Tashiro, 1988), only 0% and 15% of vaccinated orangutans have seroconverted by the same times. With the addition of 1 mg CpG ODN, this becomes 43% and 100% respectively. A synergistic response is seen even in these hyporesponders, because antibody levels and seroconversion rates are better with CpG ODN plus alum than with either adjuvant alone (FIG. 12).

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

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All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

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ataggaggtc caacgttctc 20

<210> SEQ ID NO 95

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<400> SEQUENCE: 97

tgtcgtt 7

<210> SEQ ID NO 98
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<400> SEQUENCE: 98

tgtcgct 7

We claim:

1. A composition of a synergistic combination of adjuvants, comprising:

an effective amount for inducing a synergistic adjuvant response of a combination of adjuvants, wherein the combination of adjuvants includes at least one oligonucleotide containing at least one unmethylated CpG dinucleotide and at least one non-nucleic acid adjuvant.

2. The composition of claim 1, wherein the non-nucleic acid is an adjuvant that creates a depo effect.

3. The composition of claim 2, wherein the adjuvant that creates a depo effect is selected from the group consisting of alum, emulsion based formulations, mineral oil, non-mineral oil, water-in-oil emulsions, water-in-oil-in-water emulsions, Seppic ISA series of Montanide adjuvants; MF-59; and PROVAX.

4. The composition of claim 1, wherein the non-nucleic acid adjuvant is an immune stimulating adjuvant.

5. The composition of claim 4, wherein the immune stimulating adjuvant is selected from the group consisting of saponins, PCPP polymer; derivatives of lipopolysaccharides, MPL, MDP, t-MDP, OM-174 and Leishmania elongation factor.

6. The composition of claim 1, wherein the non-nucleic acid adjuvant is an adjuvant that creates a depo effect and stimulates the immune system.

7. The composition of claim 6, wherein the adjuvant that creates a depo effect and stimulates the immune system is selected from the group consisting of ISCOMS, SB-AS2, AS2, SB-AS4, non-ionic block copolymers and SAF.

8. The composition of claim 1, wherein the composition also includes an antigen that is selected from the group consisting of peptides, polypeptides, cells, cell extracts, polysaccharides, polysaccharide conjugates, lipids, glycolipids, carbohydrates, viruses, viral extracts and antigens encoded within nucleic acids.

9. The composition of claim 8, wherein the antigen is derived from an infectious agent selected from the group consisting of a virus, bacterium, fungus and parasite.

10. The composition of claim 8, wherein the antigen is a tumor antigen.

11. The composition of claim 8, wherein the antigen is an allergen.

* * * * *

US007049302B1

(12) **United States Patent**
Kensil

(10) **Patent No.: US 7,049,302 B1**
(45) **Date of Patent: May 23, 2006**

(54) **COMPOSITIONS OF CPG AND SAPONIN ADJUVANTS AND USES THEREOF**

(75) Inventor: **Charlotte A. Kensil**, Milford, MA (US)

(73) Assignee: **Antigenics Inc.**, Lexington, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/369,941**

(22) Filed: **Aug. 6, 1999**

Related U.S. Application Data

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(51) **Int. Cl.**
A61K 48/00 (2006.01)
A61K 45/00 (2006.01)
A61K 39/00 (2006.01)

(52) **U.S. Cl.** 514/44; 424/184.1; 424/278.1

(58) **Field of Classification Search** 514/44, 514/25; 424/278.1, 184.1; 536/23.1; 435/320.1
See application file for complete search history.

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Primary Examiner—Michael Wilson
(74) *Attorney, Agent, or Firm*—Jones Day

(57) **ABSTRACT**

Vaccine compositions of immunostimulatory oligonucleotides and saponin adjuvants and antigens and the use thereof for stimulating immunity, enhancing cell-mediated immunity, and enhancing antibody production are disclosed. Also described are immune adjuvant compositions comprising immunostimulatory oligonucleotides and saponin adjuvants, as well as methods for increasing an immune response using the same.

60 Claims, 9 Drawing Sheets

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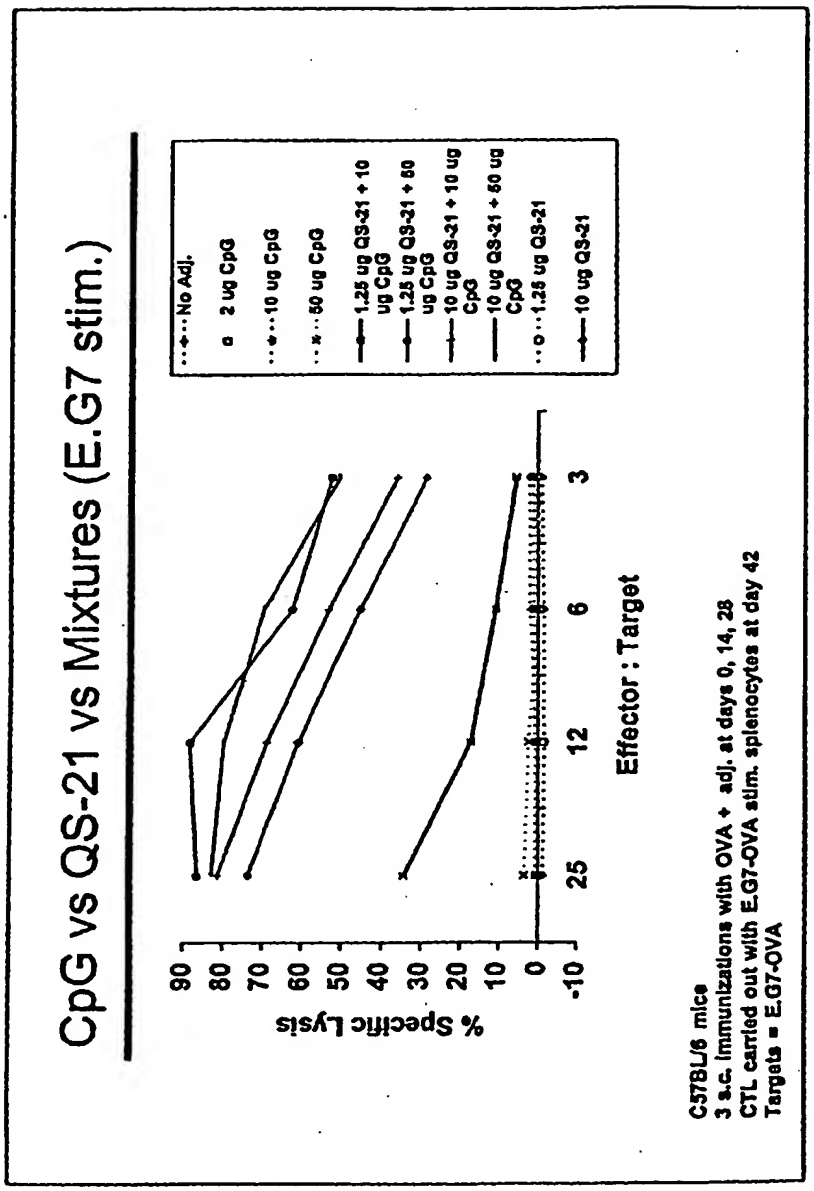

Figure 1: CTL Induced by QS-21 and CpG/QS-21

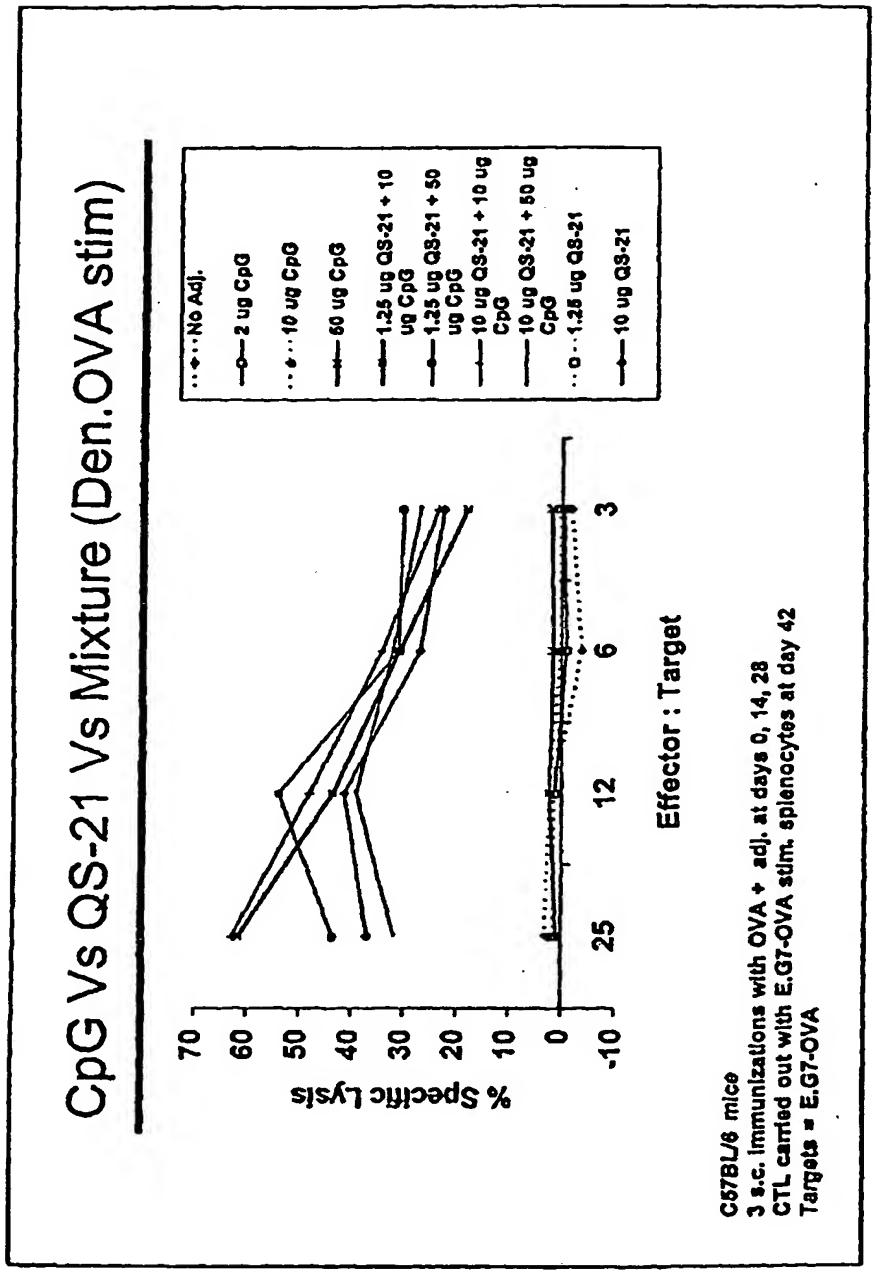

Figure 2: CTL Induced by QS-21 and CpG/QS-21

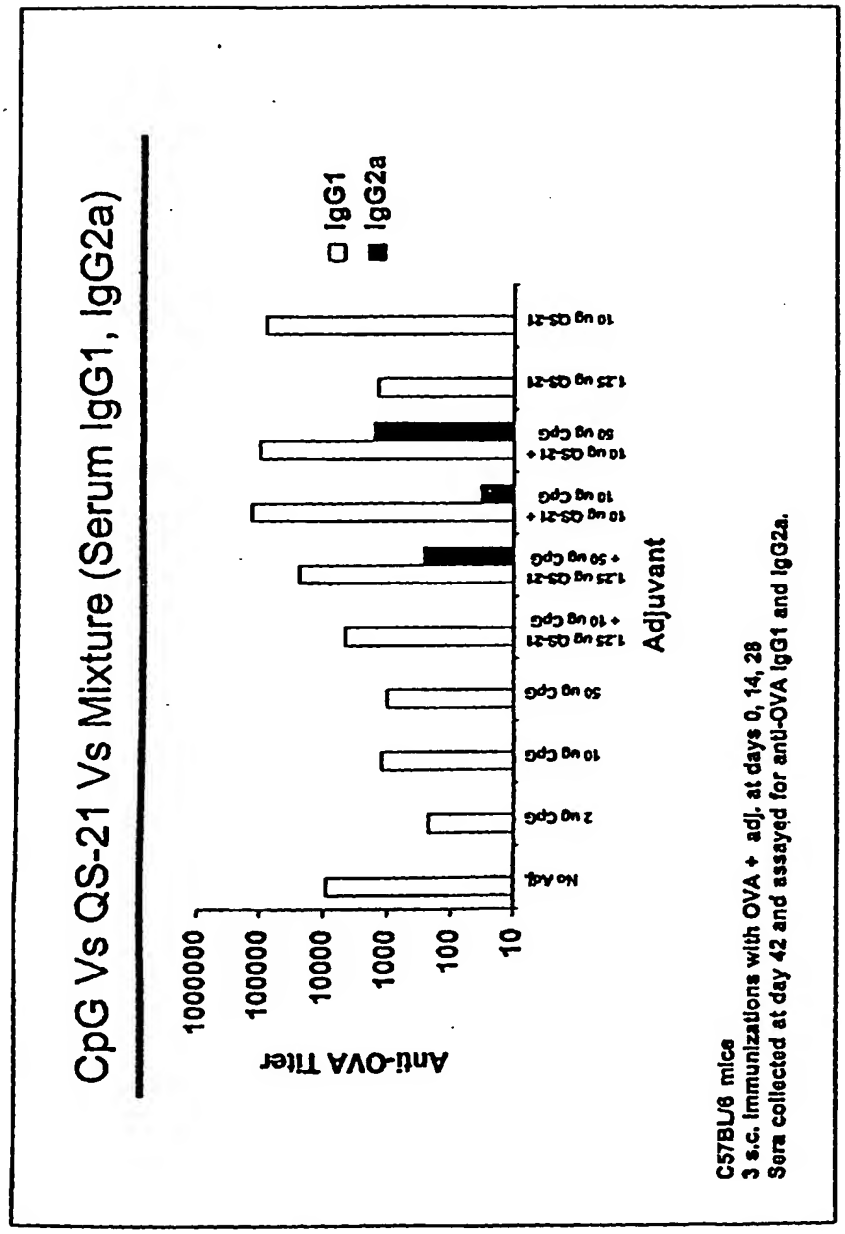

Figure 3: Antigen-specific Serum IgG1 and IgG2a

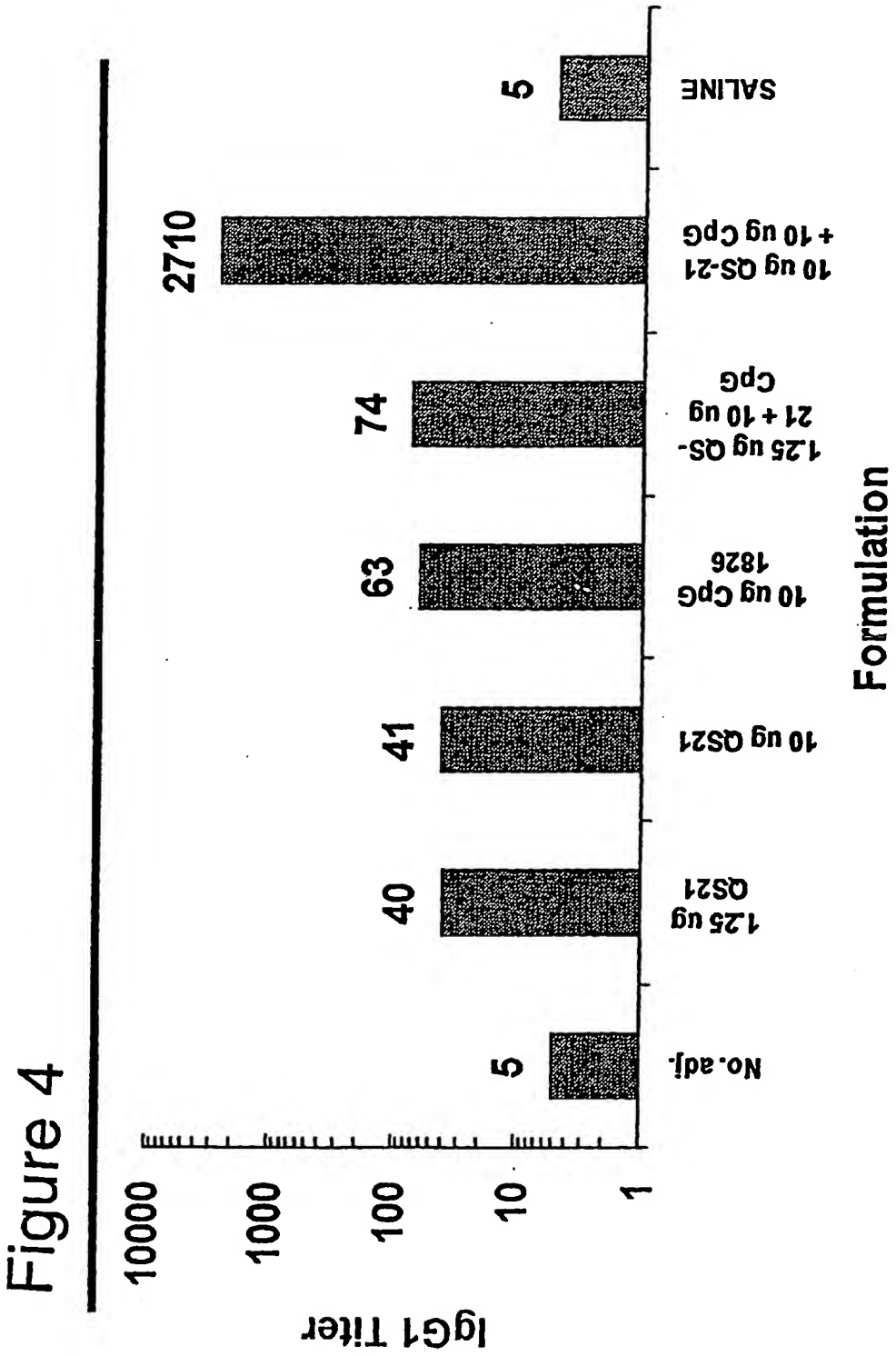
IgG1 Titer
10000
1000
100
10
1
No. adj.
5
1.25 ug QS21
40
10 ug QS21
41
10 ug CpG 1826
63
1.25 ug QS-21 + 10 ug CpG
74
10 ug QS-21 + 10 ug CpG
2710
SALINE
5
Formulation

Figure 4

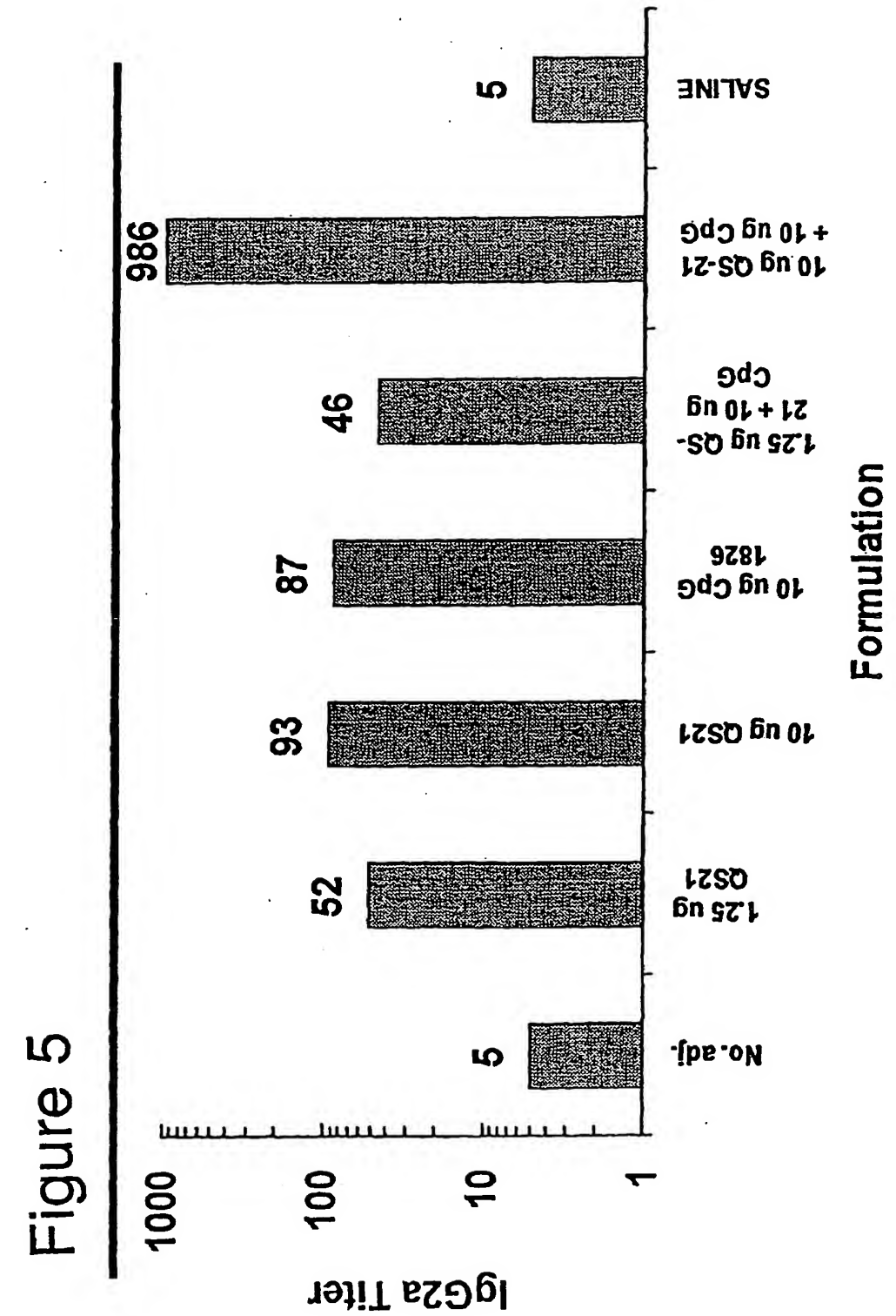
Figure 5
IgG2a Titer
1000
100
10
1
No. adj.
5
1.25 ug QS21
52
10 ug QS21
93
10 ug CpG 1826
87
1.25 ug QS-21 + 10 ug CpG
46
10 ug QS-21 + 10 ug CpG
986
SALINE
5
Formulation

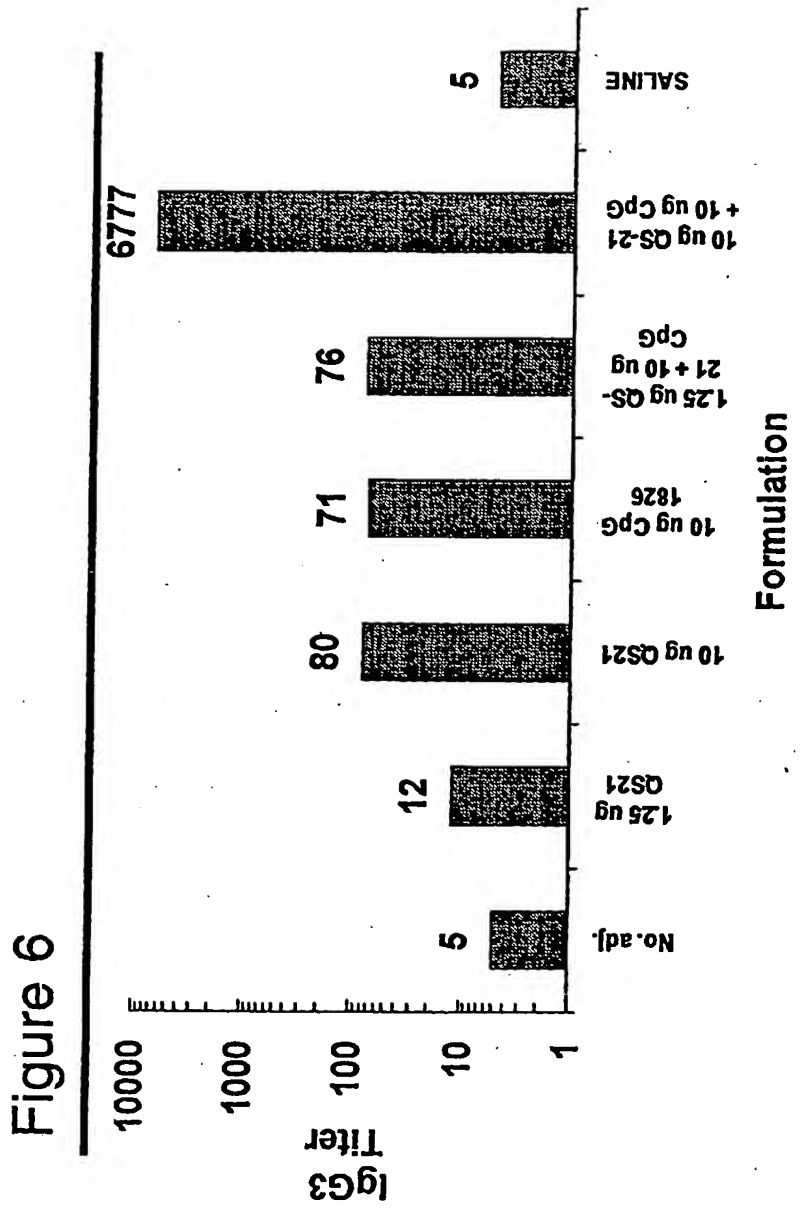
Figure 6
IgG3 Titer
10000
1000
100
10
1
No. adj.
5
125 ug QS21
12
10 ug QS21
80
10 ug CpG 1826
71
125 ug QS-21 + 10 ug CpG
76
10 ug QS-21 + 10 ug CpG
6777
SALINE
5
Formulation

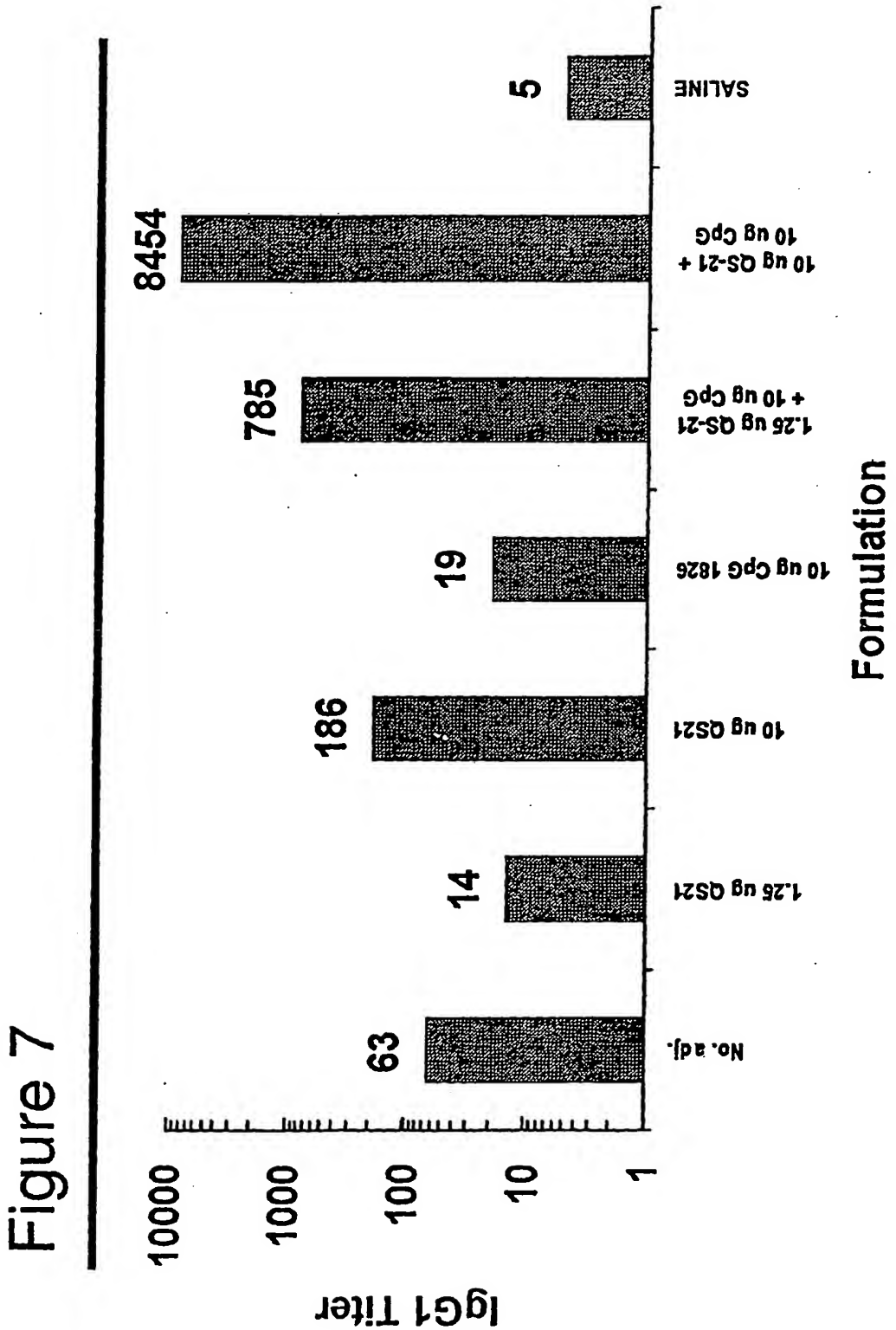
IgG1 Titer
10000
1000
100
10
1
63
14
186
19
785
8454
5
No. adj.
1.25 ug QS21
10 ug QS21
10 ug CpG 1826
1.25 ug QS-21 + 10 ug CpG
10 ug QS-21 + 10 ug CpG
SALINE
Formulation

Figure 7

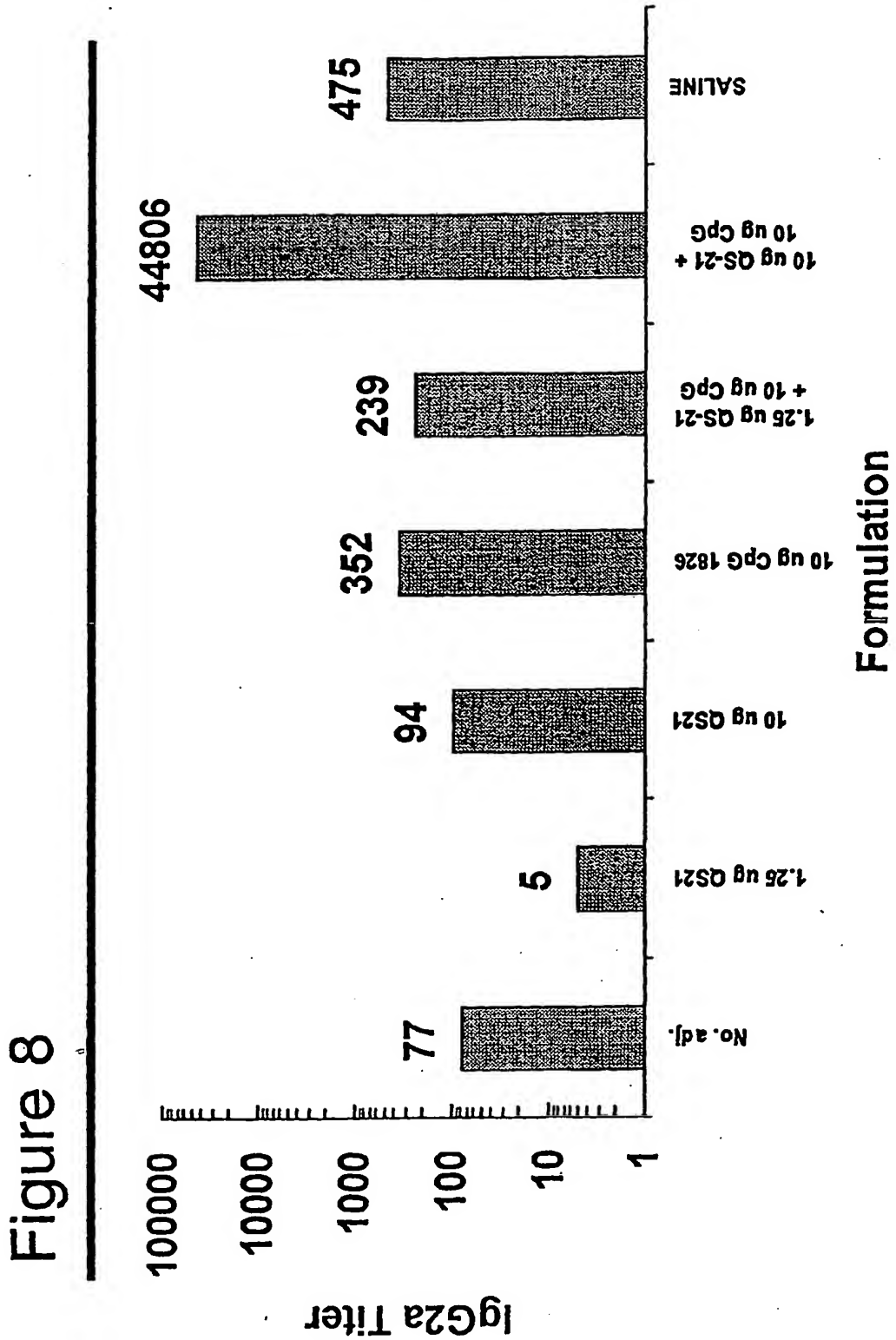
Figure 8
IgG2a Titer
100000
10000
1000
100
10
1
77
5
94
352
239
44806
475
No. adj.
1.25 ug QS21
10 ug QS21
10 ug CpG 1826
1.25 ug QS-21 + 10 ug CpG
10 ug QS-21 + 10 ug CpG
SALINE
Formulation

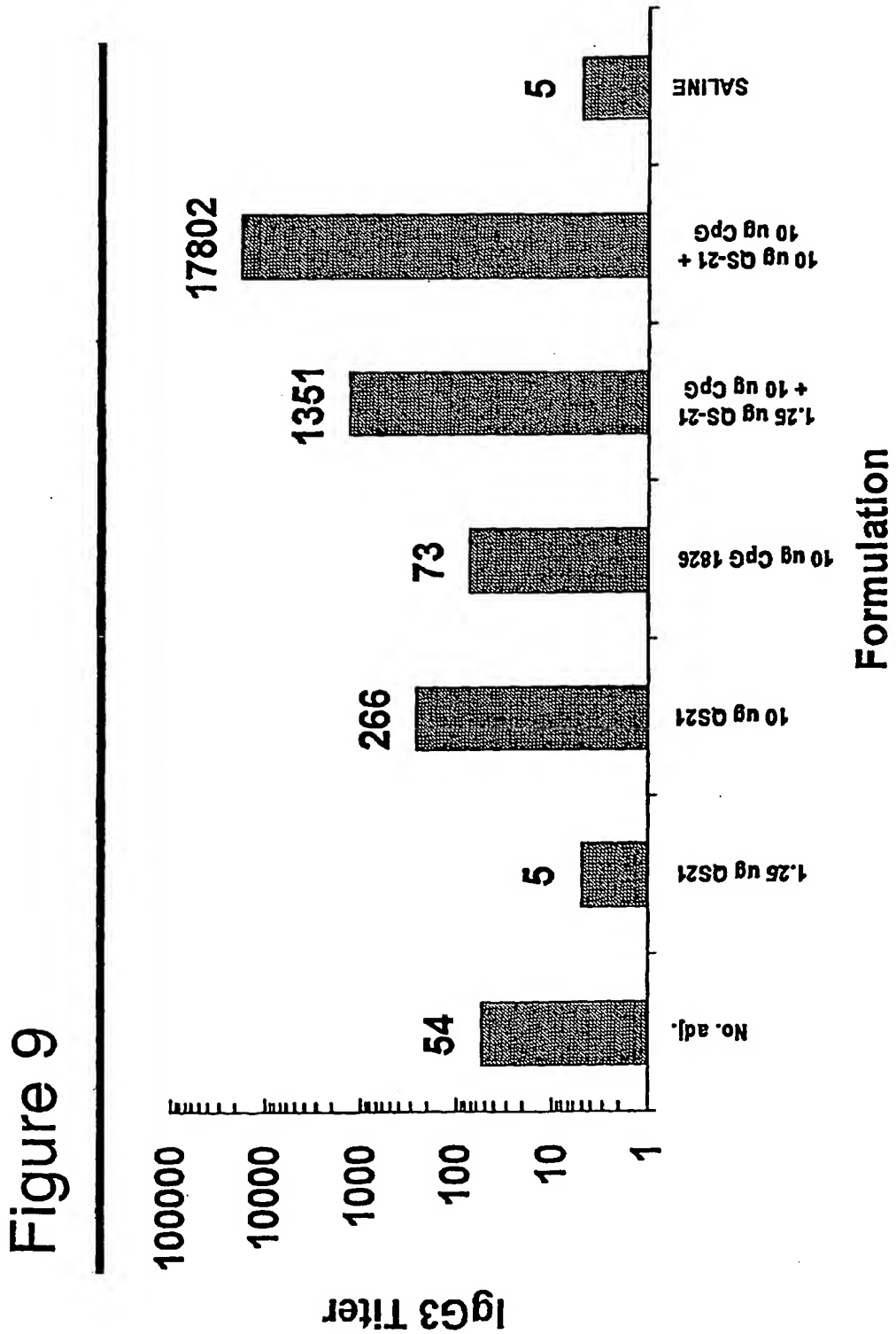
Figure 9
IgG3 Titer
100000
10000
1000
100
10
1
No. adj.
54
1.25 ug QS21
5
10 ug QS21
266
10 ug CpG 1826
73
1.25 ug QS-21 + 10 ug CpG
1351
10 ug QS-21 + 10 ug CpG
17802
SALINE
5
Formulation

COMPOSITIONS OF CPG AND SAPONIN ADJUVANTS AND USES THEREOF

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/128,608, filed Apr. 8, 1999, and of U.S. Provisional Application No. 60/095,913, filed Aug. 10, 1998, the contents of which are both incorporated herein by reference.

FIELD OF THE INVENTION

The present invention is in the field of immune adjuvants and vaccines. The compositions of the invention stimulate immunity, enhance cell-mediated immunity, and enhance antibody production.

BACKGROUND OF THE INVENTION

Adjuvant saponins have been identified and purified from an aqueous extract of the bark of the South American tree, *Quillaja saponaria* Molina. Among the 22 saponin peaks which were separable, the more predominant purified saponins have been identified as QS-7, QS-17, QS-18, and QS-21, also known as QA-7, QA-17, QA-18, and QA-21, respectively. These saponins have been substantially purified by various methods including high pressure liquid chromatography ("HPLC"), low pressure liquid silica chromatography, and hydrophilic interactive chromatography ("HILIC"). The substantially pure saponins have been found to be useful as immune adjuvants for enhancing immune responses in individuals. (Kensil, et al., U.S. Pat. No. 5,057,540; Kensil, et al., *J. Immunol.* 148:2357 (1991); Marciani, et al., *Vaccine* 9:89 (1991).)

Recently, oligonucleotides containing the unmethylated cytosine-guanine ("CpG") dinucleotide in a particular sequence context or motif have been shown to be potent stimulators of several types of immune cells in vitro. (Weiner, et al., *Proc. Natl. Acad. Sci.* 94:10833 (1997).) An immunostimulatory oligonucleotide comprising an unmethylated CpG motif is an dinucleotide within the oligonucleotide that consistently triggers an immunostimulatory response and release of cytokines. CpG motifs can stimulate monocytes, macrophages, and dendritic cells that can produce several cytokines, including the T helper 1 ("Th 1") cytokine interleukin ("IL") 12. (Carson, et al., *J. Exp. Med.* 186:1621 (1997).) This effect causes the induction of IFN-γ secretion by natural killer cells, which in turn, activates macrophages and enhances immunoglobulin isotype switching to IgG2a, a hallmark of T helper cell immunity and differentiation. (Chu, et al., *J. Exp. Med.* 186:1623 (1997).) Klinman, et al., have shown that a DNA motif consisting of an unmethylated CpG dinucleotide flanked by two 5' purines (GpA or ApA) and two 3' pyrimidines (TpC or TpT) optimally stimulated B cells to produce IL-6 and IL-12 and stimulated CD4+ T cells to produce IL-6 and IFN-γ both in vitro and in vivo. (Klinman, et al., *Proc. Natl. Acad. Sci.*, 93:2879 (1996).) Davis, et al., the contents of which are incorporated herein by reference, discovered that nucleic acids containing at least one unmethylated CpG dinucleotide may affect the immune response of a subject (Davis, et al., WO 98/40100, PCT/US98/04703).

SUMMARY OF THE INVENTION

Since immunity plays an important role in the protective response to infection with certain microbial agents, a need exists to characterize other novel adjuvants that may safely induce immunity. Such adjuvants may be potentially incorporated in future human vaccines. Surprisingly, a combination of an oligonucleotide comprising at least one unmethylated CpG dinucleotide and a saponin adjuvant was found to be a powerful stimulator of cell-mediated immunity compared to either adjuvant alone. Antibody titers (antigen-specific) in response to vaccination were significantly higher for vaccines comprising a CpG-containing oligonucleotide/saponin adjuvant combination compared to either saponin or CpG alone and represented a positive synergistic adjuvant effect. Together, these results establish that an immune adjuvant composition comprising an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide and a saponin adjuvant is a candidate adjuvant composition for vaccines to induce immunity. Accordingly, the present invention provides novel vaccine compositions which comprise an immunostimulatory oligonucleotide, a saponin adjuvant, and an antigen. Methods for increasing the immune response to an antigen by administrating the inventive vaccine compositions and/or immune adjuvant compositions are other embodiments described herein.

DESCRIPTION OF THE FIGURES

FIG. 1 depicts a graph showing the enhancement of a cell-mediated immune response by QS-21 and CpG oligonucleotide/QS-21 combination, as evidenced by the CTL induction.

FIG. 2 provides a graph showing the enhancement of a cell-mediated immune response by QS-21 and CpG oligonucleotide/QS-21 combination, as evidenced by the CTL induction.

FIG. 3 shows a bar graph of enhanced antibody production, particularly the antibody subclasses such as IgG2a that are influenced by Th 1 cytokines.

FIG. 4 shows a bar graph of IgG1 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and for combinations of QS-21 and CpG oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

FIG. 5 illustrates a bar graph of IgG2a titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

FIG. 6 provides a bar graph of IgG3 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 21 days after a first immunization given on day 0.

FIG. 7 depicts a bar graph of IgG1 titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

FIG. 8 provides a bar graph of IgG2a titers specific for pneumococcal Type 14 polysaccharide with the various formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

FIG. 9 shows a bar graph of IgG3 titers specific for pneumococcal Type 14 polysaccharide with the various

formulations and/or combinations of QS-21 and CpG oligonucleotide in mouse sera collected 14 days after a second immunization given 28 days after the first immunization.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "saponin" as used herein includes glycosidic triterpenoid compounds which produce foam in aqueous solution, have hemolytic activity in most cases, and possess immune adjuvant activity. The invention encompasses the saponin per se, as well as natural and pharmaceutically acceptable salts and pharmaceutically acceptable derivatives. The term "saponin" also encompasses biologically active fragments thereof.

The saponins of the present invention may be obtained from the tree *Quillaja saponaria* Molina. (Dalsgaard, *Acta Veterinia Scandinavica,* 69:1 (1978).) A partially purified saponin enriched extract, prepared as described by Dalsgaard, ("Quil-A") has adjuvant activity. Such an extract can be further separated. Among the 22 saponin peaks which were separable, the more predominant purified saponins have been identified as QS-7, QS-17, QS-18, and QS-21, also known as QA-7, QA-17, QA-18, and QA-21, respectively. (Kensil, et al., U.S. Pat. No. 5,057,540.) These saponins have been substantially purified by various methods including HPLC, low pressure liquid silica chromatography, and HILIC.

As described in Kensil, et al., U.S. Pat. No. 5,057,540, the contents of which are fully incorporated by reference herein, the adjuvant activity of such saponins may be determined by any of a number of methods known to those of ordinary skill in the art. The increase in antibody titer of antibody against specific antigen upon administration of an adjuvant may be used as a criteria for adjuvant activity. (Bomford, *Int. Archs. Allergy Appl. Immun.* 77:409 (1985).) Briefly, one such test involves injecting CD-1 mice intradermally with an antigen (for instance, i.e., bovine serum albumin, ("BSA")) mixed with varying amounts of the potential adjuvant. Sera was harvested from the mice two weeks later and tested by ELISA for anti-BSA antibody.

Another such test involves injecting inbred mice such as C57BL/6 or Balb/c by subcutaneous route with a protein antigen such as ovalbumin ("OVA") or a polysaccharide antigen such as pneumococcal polysaccharide, mixed with the potential adjuvant. Sera harvested form the mice after one, tow, or three immunizations could be harvested and tested by ELISA for antigen-specific antibody (total immunoglobulin) or for specific mouse IgG subclassses such as IgG1 or IgG2a. Another such test involves injecting C57BL/6 mice with OVA, harvesting spleens after one, two, or three immunizations, stimulating splenocytes with antigen, and then assaying for cytolytic T lymphocyte activity ("killing") of OVA-peptide-expressing target cells. Alternative, a proliferative response could be measured in an in vitro assay by measuring the uptake of ^{3}H-thymidine by antigen-stimulated splenocytes obtained from immunized animals.

"QS-21" designates the mixture of components QS-21-V1 and QS-21-V2 which appear as a single peak on reverse phase HPLC on Vydac C4 (5 μm particle size, 300 Å pore, 4.6 mm IDx25 cm length) in 40 mM acetic acid in methanol/water (58/42, v/v). The component fractions are referred to specifically as QS-21-V1 and QS-21-V2 when describing experiments performed on the further purified components.

According to Kensil, et al., U.S. Pat. No. 5,583,112, the contents of which are fully incorporated by reference herein, the carboxyl group on the glucuronic acid of *Quillaja saponaria* Molina can be conjugated to a protein, a peptide, or a small molecule containing a primary amine. Thus, the present invention relates to a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the modified saponin retains adjuvant activity.

The term "partially pure" means saponins partially separated from compounds normally associated with the saponin in its natural state.

The term "substantially pure" means substantially free from compounds normally associated with the saponin in its natural state and exhibiting constant and reproducible chromatographic response, elution profiles, and biologic activity. The term "substantially pure" is not meant to exclude artificial or synthetic mixtures of the saponin with other compounds.

The present invention may also employ immunostimulatory saponins isolated from other plant species. For example, a saponin from *Dolichos lablab* has been shown to be useful as an adjuvant (Katayan, et al., *Vaccine* 17:2733 (1999)).

The term "immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide" means an oligonucleotide that has been shown to activate the immune system. The immunostimulatory oligonucleotide may, preferably, comprise at least one unmethylated CpG dinucleotide. A "CpG motif" is a stretch of DNA comprising one or more CpG dinucleotides within a specified sequence. The oligonucleotide comprising the CpG motif may be as short as 4–40 base pairs in length. The immunostimulatory oligonucleotide containing the CpG motif may be a monomer or part of a multimer. Alternatively, the CpG motif may be a part of the sequence of a vector that also presents a DNA vaccine. It may be single-stranded or double-stranded. It may be prepared synthetically or produced in large scale in plasmids. One embodiment of the invention covers the immunostimulatory oligonucleotide which contains a CpG motif having the formula 5'X_1CG$X_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine or adenine. In a preferred embodiment, the CpG motif comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1; also known as "1758") or TCCATGACGTTCCTGACGTT (SEQ ID NO:2; also known as "1826").

DNA containing unmethylated CpG dinucleotide motifs in the context of certain flanking sequences has been found to be a potent stimulator of several types of immune cells in vitro. (Ballas, et al., *J. Immunol.* 157:1840 (1996); Cowdrey, et al., *J. Immunol.* 156:4570 (1996); Krieg, et al., *Nature* 374:546 (1995).) Depending on the flanking sequences, certain CpG motifs may be more immunostimulatory for B cell or T cell responses, and preferentially stimulate certain species. When a humoral response is desired, preferred immunostimulatory oligonucleotides comprising an unmethylated CpG motif will be those that preferentially stimulate a B cell response. When cell-mediated immunity is desired, preferred immunostimulatory oligonucleotides comprising at least one unmethylated CpG dinucleotide will be those that stimulate secretion of cytokines known to facilitate a CD8+ T cell response.

The immunostimulatory oligonucleotides of the invention may be chemically modified in a number of ways in order to stabilize the oligonucleotide against endogenous endonucleases. For example, the oligonucleotides may contain other than phosphodiester linkages in which the nucleotides

at the 5' end and/or 3' end of the oligonucleotide have been replaced with any number of non-traditional bases or chemical groups, such as phosphorothioate-modified nucleotides. The immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide may preferably be modified with at least one such phosphorothioate-modified nucleotide. Oligonucleotides with phosphorothioate-modified linkages may be prepared using methods well known in the field such as phosphoramidite (Agrawal, et al., *Proc. Natl. Acad. Sci.* 85:7079 (1988)) or H-phosphonate (Froehler, et al., *Tetrahedron Lett.* 27:5575 (1986)). Examples of other modifying chemical groups include alkylphosphonates, phosphorodithioates, alkylphosphorothioates, phosphoramidates, 2-O-methyls, carbamates, acetamidates, carboxymethyl esters, carbonates, and phosphate triesters. Oligonucleotides with these linkages can be prepared according to known methods (Goodchild, *Chem. Rev.* 90:543 (1990); Uhlmann, et al., *Chem. Rev.* 90:534 (1990); and Agrawal, et al., *Trends Biotechnol.* 10:152 (1992)).

The term "immune adjuvant" as used herein refers to compounds which, when administered to an individual or tested in vitro, increase the immune response to an antigen in the individual or test system to which the antigen is administered. Preferably, such individuals are mammals, and more preferably, the mammals are humans, however, the invention is not intended to be so limiting. Any animal which may experience the beneficial effects of the vaccines of the invention are within the scope of animals which may be treated according to the claimed invention. Some antigens are weakly immunogenic when administered alone, i.e., inducing no or weak antibody titers or cell-mediated immune response. An immune adjuvant may enhance the immune response of the individual by increasing antibody titers and/or cell-mediated immunity. The adjuvant effect may also lower the dose of the antigen effective to achieve an immune response in the individual.

In a first aspect of the invention, an immune adjuvant composition comprising a saponin adjuvant and an immunostimulatory oligonucleotide may be administered. More preferably, such immune adjuvant composition may increase the immune response to an antigen in an individual or a test system to which the antigen is administered. Preferably, the saponin adjuvant is a saponin from *Quillaja saponaria* Molina. More preferably, the saponin adjuvant is a partially pure or substantially pure saponin from *Quillaja saponaria* Molina. Preferably, the partially pure saponin may comprise QS-7, QS-17, QS-18, and/or QS-21 and may comprise other saponins. Preferably, the substantially pure saponin adjuvant is QS-7, QS-17, QS-18, or QS-21. Most preferably, the substantially pure saponin adjuvant is QS-21. Alternatively, the immune adjuvant composition may comprise more than one substantially pure saponin adjuvant with the immunostimulatory oligonucleotide. In a further preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. The immunostimulatory oligonucleotide, preferably, compries at least one unmethylated CpG dinucleotide. The CpG dinucleotide is preferably a monomer or multimer. Another preferred embodiment of the CpG motif is as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the immune adjuvant composition is directed to the immunostimulatory oligonucleotide, wherein the immunostimulatory oligonucleotide is modified. The particular modification may comprise at least one phosphorothioate-modified nucleotide. Further, the immunostimulatory oligonucleotide having at least one unmethylated CpG dinucleotide may comprise a CpG motif having the formula 5'X_1CG$X_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. The CpG motif may preferentially be TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1] or TCCATGACGTTCCTGACGTT [SEQ ID NO.:2]

In a second aspect, the invention is directed to a method for increasing the immune response to an antigen in an individual or a test system to which the antigen is administered comprising administering an effective amount of an immune adjuvant composition comprising a saponin adjuvant and an immunostimulatory oligonucleotide further. Preferably, the saponin adjuvant is a saponin from *Quillaja saponaria* Molina. More preferably, the saponin adjuvant is a partially pure or a substantially pure saponin from *Quillaja saponaria* Molina. The method may also embody an immune adjuvant composition comprising more than one substantially pure saponin adjuvant and immunostimulatory oligonucleotide. The substantially pure saponin adjuvant is preferably QS-7, QS-17, QS-18, or QS-21. Most preferably, the substantially pure saponin adjuvant is QS-21. In a further preferred embodiment, the saponin adjuvant may cover a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. In a preferred embodiment of the method, the immunostimulatory oligonucleotide comprises at least one unmethylated CpG dinucleotide. The CpG motif is preferably a monomer or a multimer. Another preferred embodiment of the method includes the CpG motif as a part of the sequence of a vector that presents a DNA vaccine. Yet another embodiment is directed to the method wherein the immunostimulatory oligonucleotide comprises at least one unmethylated CpG dinucleotide, and wherein furthermore, the immunostimulatory oligonucleotide may be chemically modified to stabilize the oligonucleotide against endogenous endonucleases. The modification may comprise at least one phosphorothioate-modified nucleotide. Further, the method may be directed, in part, to the immunostimulatory oligonucleotide having at least one unmethylated CpG dinucleotide comprising a CpG motif having the formula 5'X_1CG$X_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. In another preferred method, the unmethylated CpG motif is TCTCCCATCGTGCGCCAT [SEQ ID NO.:1] or TCCATGACGTTCCTGACGTT [SEQ ID NO.:2]

The term "vaccine composition" herein refers to a composition capable of producing an immune response. A vaccine composition, according to the invention, would produce immunity against disease in individuals. The combination of saponin and immunostimulatory oligonucleotide of the present invention may be administered to an individual to enhance the immune response to any antigen. Preferably, the vaccine composition stimulates immunity. More preferably, the vaccine composition enhances antibody production to an antigen and enhances a cell-mediated immune response to an antigen.

The vaccine composition of the invention may enhance antibody production to an antigen in a positive synergistic manner. The synergistic adjuvant effect of the immuno-

stimulatory oligonucleotide and the saponin adjuvant described herein may be shown in a number of ways. For example, a synergistic adjuvant effect may be demonstrated as an increase in the maximum expected immune response. One may expect an additive effect of combining two adjuvants. Specifically, if one adjuvant, used at optimum doses, produces "X" and the other adjuvant, also used at optimum doses, produces "Y" antibody, then the combination may be expected to produce "X+Y" if the result is additive and not synergistic. A maximum level of response that is considerably higher than "X+Y" would be considered a synergistic effect and would be unexpected. A second indication of synergism would be the appearance of a substantial adjuvant effect at doses that are normally not expected to produce an adjuvant effect. A third indication of synergism would be the appearance of an immune response with earlier kinetics than expected for either adjuvant alone.

Further, typical antigens suitable for the enhanced immune response include antigens derived from any of the following: viruses, such as influenza, feline leukemia virus, feline immunodeficiency virus, HIV-1, HIV-2, rabies, measles, hepatitis B, or hoof and mouth disease; bacteria, such as anthrax, diphtheria, Lyme disease, pneumococcus, or tuberculosis; or protozoans, such as *Babeosis bovis* or Plasmodium. The antigen may preferably be a protein, a peptide, a polysaccharide, a lipid, a glycolipid, a phospholipid, or a nucleic acid encoding the antigenic protein or peptide of interest. The antigens may be purified from a natural source, synthesized by means of solid phase synthesis, or may be obtained by means of genetic engineering.

Accordingly, in a third aspect, the invention also encompasses a vaccine composition comprising a saponin adjuvant, an immunostimulatory oligonucleotide, and an antigen. The saponin adjuvant may be partially pure or substantially pure saponin from *Quillaja saponaria* Molina. The vaccine compositions may also comprise more than one partially pure or substantially pure saponin adjuvant, an immunostimulatory oligonucleotide further comprising at least one unmethylated CpG motif, and an antigen. Preferably, the partially pure saponin adjuvant comprises QS-7, QS-17, QS-18, and/or QS-21 and may comprise other saponins. Preferably, the substantially pure saponin adjuvant is QS-7, QS-17, QS-18, or QS-21. A further preferred embodiment encompasses saponin adjuvants wherein a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. Most preferably, the partially pure or substantially pure saponin adjuvant in the vaccine composition is QS-21. The immunostimulatory oligonucleotide may preferably comprise at least one unmethylated CpG dinucleotide. The CpG motif may preferably be a monomer or a multimer. Another preferred embodiment of the CpG motif is as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the vaccine composition described herein is directed to the immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide comprises a chemical modification. More particularly, the immunostimulatory oligonucleotide may be modified with at least one phosphorothioate-modified nucleotide. Further, the immunostimulatory oligonucleotide having at least one unmethylated CpG dinucleotide of the vaccine composition comprises a CpG motif having the formula 5'X_1CG$X_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. The unmethylated CpG motif according to this aspect of the invention may preferentially comprise TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1]or TCCATGACGTTCCTGACGTT [SEQ ID NO.:2]

A fourth aspect of the invention encompasses a method of stimulating immunity to an antigen in an individual comprising administering an effective amount of a vaccine composition comprising an antigen, a partially pure or substantially pure saponin adjuvant, and an immunostimulatory oligonucleotide. The method also embodies a vaccine composition comprising more than one partially pure or substantially pure saponin adjuvant, an immunostimulatory oligonucleotide, and an antigen. Preferably, the partially pure saponin adjuvant comprises QS-7, QS-17, QS-18, and/or QS-21 and may comprise other saponins. Preferably, the substantially pure saponin adjuvant comprises QS-7, QS-17, QS-18, or QS-21. Most preferably, according to this method, the partially pure or substantially pure saponin adjuvant is QS-21. The saponin adjuvant may preferably be a chemically modified saponin adjuvant or a fraction thereof obtainable from a crude *Quillaja saponaria* Molina extract, wherein the chemically modified saponin or fraction thereof comprises at least one of QS-17, QS-18, QS-21, QS-21-V1, and QS-21-V2, and wherein the chemically modified saponin retains adjuvant activity. Preferably, the method comprises administering an immunostimulatory oligonucleotide which further comprises at least one unmethylated CpG dinucleotide. The CpG dinucleotide therein is a monomer or a multimer. Another preferred embodiment of the method includes the CpG motif as a part of the sequence of a vector that also presents a DNA vaccine. Yet another embodiment of the method disclosed herein is directed to the immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide, wherein the immunostimulatory oligonucleotide may be chemically modified to increase its stability to endogenous endonucleases. Such a modification may comprise at least one phosphorothioate-modified nucleotide. Further, the immunostimulatory oligonucleotide having at least one unmethylated CpG dinucleotide may comprise a CpG motif having the formula 5'X_1CG$X_2$3', wherein at least one nucleotide separates consecutive CpGs, and wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine. In another preferred embodiment, the unmethylated CpG motif is TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1] or TCCATGACGTTCCTGACGTT [SEQ ID NO.:2]

Other useful methods for the vaccine composition include enhancing antibody production to an antigen and enhancing cell-mediated immunity. More preferably, the vaccine composition enhances antibody production to an antigen and enhances a cell-mediated immunity. Most preferably, the vaccine composition enhances antibody production to an antigen in a positive synergistic manner.

Administration of the compositions of the present invention may be by parenteral, intravenous, intramuscular, subcutaneous, intranasal, oral, mucosal, or any other suitable means. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the antigen administered. The initial dose may be followed up with a booster dosage after a period of about four weeks to enhance the immunogenic response. Further booster dosages may also be administered. The composition may be given as a single injection of a mixed formulation of saponin, oligonucleotide, and antigen or as separate injections given at the same site within a short period of time (i.e., 0–2 days).

The effective compositions of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert acceptable carrier may preferably be used, such as saline, or PBS, or any such acceptable carrier in which the compositions of the present invention have suitable solubility properties for use of the present invention.

EXAMPLES

A well-established animal model was used to assess whether formulations of CpG oligonucleotide and QS-21 together could function as an immune adjuvant. In brief, experiments were set up to compare QS-21 to the recently reported adjuvant CpG motif. A CpG sequence (e.g., 1758), reported to serve as an adjuvant for a B-cell lymphoma idiotype-KLH vaccine in mice, was selected. One experiment evaluated whether the CpG motif, alone or in combination with QS-21, can serve as an adjuvant for a subunit vaccine, e.g., OVA, in mice in inducing CTL responses. This work included a dose range experiment with CpG to determine the optimum dose.

In addition to comparing CpG and QS-21 as adjuvants, a second experiment combining CpG oligonucleotide with suboptimal doses of QS-21 (e.g., 1.25 μg) was conducted to assess whether CpG oligonucleotide can affect the adjuvant effect of QS-21.

Also, an experiment was performed to determine whether the CpG and QS-21 combination could enhance antibody production, specifically the isotype profile of a antigen-specific antibody response.

Finally, a series of experiments were performed to determine whether a combination of CpG oligonucleotide and saponin would enhance antibody production in a positive synergistic manner. This work used vaccine formulations of pneumococcal Type 14 polysaccharide and QS-21 and CpG oligonucleotide and evaluated specific antibody titers harvested from mice on days 21 and 42 after immunization on days 0 and 28. Another CPG sequence (e.g., 1826), reported to serve as an adjuvant for hen egg lysozyme in mice, was selected.

The experiments were done using materials from the following suppliers: OVA, Grade VI (Sigma); pneumococcal Type 14 polysaccharide (ATCC); QS-21 (Aquila); CpG oligonucleotides included the phosphorothiate-modified sequence 1758 TCTCCCAGCGTGCGCCAT [SEQ ID NO.: 1] and phosphorothiate-modified sequence 1826 TCCATGACGTTCCTGACGTT [SEQ ID NO.:2] (Life Technologies (Gibco)).

Example 1

CTL Induced by OS-21 and CpG/OS-21

C57BL/6 mice (5 per group, female, 8–10 weeks of age) were immunized by subcutaneous route at days 1, 15, and 29. The vaccines were 25 μg OVA antigen plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. The CpG motif used in this experiment was a phosphorothioate-modified oligonucleotide 1758 with a sequence of TCTCCCAGCGTGCGCCAT [SEQ ID NO.:1] (Weiner, et al., *Proc. Natl. Acad. Sci.* 94:10833 (1997).) Splenocytes were removed at day 42 for use as effector cells in the CTL assay. They were stimulated in vitro for 6 days with mitomycin C-treated E.G7-OVA cells and then used in a standard ^{51}Cr release CTL assay. E.G7-OVA cells (loaded with ^{51}Cr) were used as target cells. The background lysis of EL4 cells (not transfected by OVA) was subtracted from the lysis of E.G7-OVA cells to obtain a percent (%) antigen-specific lysis.

The results, as shown in FIG. 1, indicate that no lysis was observed in the absence of adjuvant, with any CpG dose, or with 1.25 μg of QS-21 (suboptimal dose). However, the suboptimal dose of QS-21, in combination with CpG, induced significant CTL. The results show a substantial adjuvant effect at doses that are normally not expected to produce such an adjuvant effect. This positive synergistic effect was most notable at the higher dose of CpG (50 μg). The adjuvant effect was comparable to that achieved with the optimal 10 μg QS-21 control.

Example 2

CTL Induced by OS-21 and CpG/OS-21

Splenocytes from mice immunized as described in FIG. 1 were used in a CTL assay. Splenocytes were stimulated in vitro with denatured OVA for six days prior to use in the CTL assay. The assay was carried out against E.G7-OVA cells as described in Example 1.

As evident from the results in FIG. 2, no lysis was observed in the absence of adjuvant, with any CpG dose, or with 1.25 μg of QS-21 (suboptimal dose). However, the suboptimal dose of QS-21, in combination with CpG, induced significant CTL (comparable to the optimal 10 μg QS-21 control). The results illustrate the positive synergism between the CpG and the QS-21 that was unexpected at a suboptimal dose.

Example 3

Antigen-specific Serum IgG1 and IgG2a

Serum titers to OVA were determined by EIA on sera collected on day 42 from the mice immunized as described in Example 1. IgG subclass IgG1 and IgG2a titers were determined for individual mice (5 mice per group) and are plotted as a geometric mean titer. The IgG1 titers were highest in groups receiving QS-21 alone (at the 10 μg dose) or 10 μg QS-21 in combination with either 10 or 50 μg (approximate 10 fold enhancement over the unadjuvanted group) as seen in FIG. 3. The IgG2a response was not detectable in any groups except for the combination of 10 μg QS-21 (optimal dose) with 10 or 50 μg CpG and the combination of 1.25 μg QS- 21 (suboptimal dose) with 50 μg CpG. IgG2a was not detected with any CpG dose used alone, with any QS-21 dose used alone, or in the unadjuvanted group.

Example 4

Antibody Induced by OS-21 and OS-21/CpG to Pneumococcal Polysaccharide Antigen

BALB/c mice (5 mice per group, female, 8–10 weeks of age) were immunized by subcutaneous route at day 0 only or at days 0 and 28. The vaccines were 0.5 μg pneumococcal Type 14 polysaccharide plus the indicated doses of adjuvant in a total volume of 0.2 ml phosphate-buffered saline. The immunostimulatory motif CpG used in this experiment was a phosphorothioate-modified oligonucleotide 1826 with a sequence of TCCATGACGTTCCTGACGTT [SEQ ID NO.: 2] (Chu, et al., *Exp. Med.* 186:1623–1631 (1997)). QS-21

was used at a dose of 1.25 μg or 10 μg. CpG ODN 1826 was used at a dose of only 10 μg.

Sera from mice receiving a single immunization was collected at day 21. Sera from mice receiving 2 immunizations was collected at day 42. Antibody titers specific for Type 14 polysaccharide was determined on the sera. IgG subclasses IgG1, IgG2a, and IgG3 were determined for an equivolume sera pool from the mice in each group. After a single immunization, IgG1 titers were 66 fold higher for the 10 μg QS-21/10 μg CpG combination than for QS-21 alone and were 43 fold higher than for CpG alone (FIG. 4). IgG2a titers were 11 fold higher for the 10 μg QS-21/CpG combination than for either QS-21 alone or CpG alone (FIG. 5). IgG3 titers were 85 fold higher for the 10 μg QS-21/CpG combination than for QS-21 alone and were 95 fold higher than for CpG alone (FIG. 6).

After two immunizations, IgG1 titers were 46 fold higher for the 10 μg QS-21/CpG combination than for QS-21 alone and were 444 fold higher than for CpG alone (FIG. 7). IgG2a titers were 476 fold higher for the 10 μg QS-21/CpG combination than for QS-21 alone and were 127 fold higher than for CpG alone (FIG. 5). IgG3 titers were 67 fold higher for the 10 μg QS-21/CpG combination than for QS-21 alone and were 243 fold higher than for CpG alone (FIG. 9). The enhancement of these titers shows that this is a positive synergistic effect and is not simply an additive adjuvant effect of combining these two adjuvants. In addition, the combination of low doses of QS-21 (1.25 μg) with 10 μg CpG also produced IgG1 and IgG3 titers after two immunizations that were higher than those produced by either 1.25 μg QS-21 alone, 10 μg QS-21 alone, or 10 μg CpG alone.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.

wherein the immunostimulatory oligonucleotide is not a part of a DNA vaccine vector, and

wherein the saponin and immunostimulatory oligonucleotide have a synergistic adjuvant effect.

2. The immune adjuvant composition as claimed in claim 1, wherein the saponin comprises a substantially pure saponin.

3. The immune adjuvant composition as claimed in claim 2, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

4. The immune adjuvant composition as claimed in claim 3, wherein the substantially pure saponin is QS-21.

5. The immune adjuvant composition as claimed in claim 1 or 4, wherein the immunostimulatory oligonucleotide comprises more than one unmethylated CpG dinucleotide.

6. The immune adjuvant composition as claimed in claim 1 or 4, wherein the immunostimulatory oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

7. The immune adjuvant composition as claimed in claim 1 or 4, wherein the immunostimulatory oligonucleotide comprises at least one phosphorothioate modified nucleotide.

8. The immune adjuvant composition as claimed in claim 1, wherein the immunostimulatory oligonucleotide comprises a CpG motif having the formula 5'X_1CG$X_2$3', wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

9. The immune adjuvant composition as claimed in claim 1 or 4, wherein the immunostimulatory oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1).

10. An immune adjuvant composition comprising

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 2

<210> SEQ ID NO 1
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Quillaja saponaria

<400> SEQUENCE: 1

tctcccagcg tgcgccat 18

<210> SEQ ID NO 2
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Quillaja saponaria

<400> SEQUENCE: 2

tccatgacgt tcctgacgtt 20

I claim:

1. An immune adjuvant composition comprising

(a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from *Quillaja saponaria*; and

(b) an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide,

(a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from *Quillaja saponaria*; and

(b) an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide,

wherein the saponin is substantially pure, and the saponin is QS-7, QS-17 or QS-18, and

wherein the saponin and immunostimulatory oligonucleotide have a synergistic adjuvant effect.

11. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 10 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

12. An immune adjuvant composition comprising

(a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from *Quillaja saponaria*; and

(b) an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide,

wherein the immunostimulatory oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester, and

wherein the saponin and immunostimulatory oligonucleotide have a synergistic adjuvant effect.

13. The immune adjuvant composition as claimed in claim 12, wherein the immunostimulatory oligonucleotide comprises at least one phosphorothioate modified nucleotide.

14. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 12 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

15. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 13 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

16. An immune adjuvant composition comprising

(a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from *Quillaja saponaria*; and

(b) an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide,

wherein the immunostimulatory oligonucleotide comprises TCTCCCAGCGTFCGCCAT (SEQ ID NO:1), and,

wherein the saponin and immunostimulatory oligonucleotide have a synergistic adjuvant effect.

17. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 16 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

18. An immune adjuvant composition comprising

(a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from *Quillaja saponaria*; and

(b) an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide,

wherein the immunostimulatory oligonucleotide comprises TCCATGACGTTCCTGACGTT (SEQ ID NO:2), and

wherein the saponin and immunostimulatory oligonucleotide have a synergistic adjuvant effect.

19. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 18 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

20. An immune adjuvant composition comprising

(a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from *Quillaja saponaria*; and

(b) an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide, wherein the immunostimulatory oligonucleotide is 4–40 bases in length, and

wherein the saponin and immunostimulatory oligonucleotide have a synergistic adjuvant effect.

21. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 20 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

22. An immune adjuvant composition comprising

(a) a saponin possessing immune adjuvant activity, wherein the saponin (i) is derived from *Quillaja saponaria* and (ii) is a chemically modified saponin; and

(b) an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide,

wherein the saponin and immunostimulatory oligonucleotide have a synergistic adjuvant effect.

23. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 22 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

24. The composition of claim 1, wherein the saponin is a chemically modified saponin.

25. The immune adjuvant composition as claimed in claim 1 or 4, wherein the immunostimulatory oligonucleotide comprises TCCATGACGTTCCTGACGTT (SEQ ID NO:2).

26. A method for inducing an immune response in an individual to an antigen comprising (1) administering an amount of the immune adjuvant composition as claimed in claim 1 to the individual; and (2) administering a nucleic acid molecule comprising a nucleotide sequence encoding the antigen to the individual, wherein (1) and (2) induce an immune response in the individual to the antigen.

27. The method as claimed in any of claims 14, 15, 17, 19, 21, 23, or 26, wherein the saponin comprises is a substantially pure saponin.

28. The method as claimed in claim 27, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

29. The method as claimed in claim 28, wherein the substantially pure saponin is QS-21.

30. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the immunostimulatory oligonucleotide comprises more than one unmethylated CpG dinucleotide.

31. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the immunostimulatory oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

32. The method as claimed in any of claims 11, 17, 19, 21, 23, or 26, wherein the immunostimulatory oligonucleotide comprises at least one phosphorothioate modified nucleotide.

33. The method as claimed in any of claims 11, 14, 15, 17, 19, 21, 23, or 26, wherein the immunostimulatory oligonucleotide comprises a CpG motif having the formula $5'X_1CGX_2 3'$, wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

34. The method as claimed in any of claims 11, 14, 15, 21, 23, or 26, wherein the immunostimulatory oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or TCCATGACGTTCCTGACGTT (SEQ ID NO:2).

35. The method as claimed in any of claims 11, 14, 15, 21, 19, 21, 23, or 26, wherein the individual is an animal.

36. The method as claimed in claim 35, wherein the animal is a mammal.

37. The method as claimed in any of claims 11, 14, 15, 21, 19, 21, 23, or 26, wherein the individual is a human.

38. A vaccine composition comprising

(a) a saponin possessing immune adjuvant activity, wherein the saponin is derived from *Quillaja saponaria;*

(b) an immunostimulatory oligonucleotide comprising at least one unmethylated CpG dinucleotide; and

(c) a nucleic acid molecule comprising a nucleotide sequence encoding an antigen, wherein the nucleotide sequence is operatively linked to a promoter,

wherein the immunostimulatory oligonucleotide is not a part of the nucleic acid molecule comprising the nucleotide sequence encoding the antigen.

39. The vaccine composition as claimed in claim 38, wherein the saponin is a substantially pure saponin.

40. The vaccine composition as claimed in claim 39, wherein the substantially pure saponin is QS-7, QS-17, QS-18, or QS-21.

41. The vaccine composition as claimed in claim 40, wherein the substantially pure saponin is QS-21.

42. The vaccine composition as claimed in claim 38, wherein the immunostimulatory oligonucleotide comprises more than one unmethylated CpG dinucleotide.

43. The vaccine composition as claimed in claim 38, wherein the immunostimulatory oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

44. The vaccine composition as claimed in claim 38, wherein the immunostimulatory oligonucleotide comprises at least one phosphorothioate modified nucleotide.

45. The vaccine composition as claimed in claim 38, wherein the immunostimulatory oligonucleotide comprises a CpG motif having the formula $5'X_1CGX_2 3'$, wherein X_1 is adenine, guanine, or thymine, and X_2 is cytosine, thymine, or adenine.

46. The vaccine composition as claimed in claim 38 or 41, wherein the immunostimulatory oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or TCCATGACGTTCCTGACGTT (SEQ ID NO:2).

47. The method of any of claims 11, 17, 19, 23, or 26, wherein the nucleic acid molecule encoding the antigen is administered to the individual concurrently with the immune adjuvant composition.

48. The method of any of claims 14, 15, or 21, wherein the nucleic acid molecule encoding the antigen is administered to the individual concurrently with the immune adjuvant composition.

49. The method as claimed in any of claims 14, 15, 21, 23, and 26, wherein the saponin is substantially pure, wherein the saponin is QS-21, and wherein the immunostimulatory oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1or TCCATGACGTTCCTGACGTT (SEQ ID NO:2).

50. The immune adjuvant composition as claimed in claim 12 or 20, wherein the saponin is chemically modified.

51. The immune adjuvant composition as claimed in claim 12, 20 or 22, wherein the immunostimulatory oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or TCCATGACGTTCCTGACGTT (SEQ ID NO:2).

52. The immune adjuvant composition as claimed in claim 12 or 22, wherein the saponin is substantially pure.

53. The immune adjuvant composition as claimed in claim 52, wherein the saponin is QS-21.

54. The immune adjuvant composition as claimed in claim 53, wherein the immunostimulatory oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or TCCATGACGTTCCTGACGTT (SEQ ID NO:2).

55. The immune adjuvant composition as claimed in claim 20, wherein the saponin is substantially pure.

56. The immune adjuvant composition as claimed in claim 55, wherein the saponin is QS-21.

57. The immune adjuvant composition as claimed in claim 20 or 56, wherein the immunostimulatory oligonucleotide comprises at least one chemical group selected from the group consisting of phosphorothioate, alkylphosphonate, phophorodithioate, alkylphosphorothioate, phosphoramidate, 2-O-methyl, carbamate, acetamidate, carboxymethyl ester, carbonate, and phosphate triester.

58. The immune adjuvant composition as claimed in claim 56, wherein the immunostimulatory oligonucleotide comprises TCTCCCAGCGTGCGCCAT (SEQ ID NO:1) or TCCATGACGTTCCTGACGTT (SEQ ID NO:2).

59. The immune adjuvant composition as claimed in claim 16 or 18, wherein the saponin is substantially pure.

60. The immune adjuvant composition as claimed in claim 59, wherein the saponin is QS-21.

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